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Art Unit 1636

Attn: Mail Stop Appeal Brief - Patents

Re: U.S. Utility Patent Application
Application No. 10/035,216; Filed: January 4, 2002
For: **Viral Delivery System for Infectious Transfer of Large Genomic DNA Inserts**
Inventors: Chiocca *et al.*
Our Ref: 0609.5050005/TJS/KRM

Sir:

Transmitted herewith for appropriate action are the following documents:

1. Brief on Appeal Under 37 C.F.R. § 41.37, along with Exhibits 1-4;

2. Credit Card Payment Form (PTO-2038) in the amount of **$250.00** to cover the Brief on Appeal (small entity); and

3. Return postcard.

It is respectfully requested that the attached postcard be stamped with the date of filing of these documents, and that it be returned to our courier. In the event that extensions of time are necessary to prevent abandonment of this patent application, then such extensions of time are hereby petitioned.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036.

Respectfully submitted,

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KRM/pcd
Enclosures

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

CHIOCCA *et al.*.

Appl. No. 10/035,216

Filing date: January 4, 2002

For: **Viral Delivery System for Infectious Transfer of Large Genomic DNA Inserts**

Confirmation No.: 3452

Art Unit: 1636

Examiner: Sullivan, D. M.

Atty. Docket: 0609.5050005/TJS/KRM

Brief on Appeal Under 37 C.F.R. § 41.37

Mail Stop Appeal Brief - Patents

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

A Notice of Appeal from the final rejection of claims 40-45 and 47-57 was filed on August 3, 2006. Appellants hereby file this Appeal Brief, together with the required brief filing fee and any necessary extension of time fees.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Table of Contents

Table of Contents i

I. Real Party In Interest 1

II. Related Appeals and Interferences 1

III. Status of Claims 1

IV. Status of Amendments 1

V. Summary of Claimed Subject Matter 1

A. Overview of Claimed Subject Matter 1

B. The Subject Matter Defined by Independent Claim 40 and Support in the Specification Therefor 3

VI. Grounds of Rejection to be Reviewed on Appeal 4

VII. Argument 4

A. Legal Principles Relating to Nonobviousness 4

B. Ground 1 of the Rejection: Kim in View of Wang and Woodfield 5

1. Summary of Cited References 5

(a) Kim (Exhibit 1) 5

(b) Wang (Exhibit 2) 5

(c) Woodfield (Exhibit 3) 6

2. Summary of the Examiner's Basis for the Rejection 6

3. A Person of Ordinary Skill in the Art Would Not Have Been Motivated to Modify and/or Combine the Cited References 7

(a) Kim Does Not Suggest a Need to Improve The Efficiency With Which Genomic DNA is Introduced Into Cells 7

(b) The Examiner's Asserted Advantage of Combining the References Could Have Been Achieved Most Directly Using the Vector of Wang Without Any Modification or Combination 12

4. The Examiner's Basis for the Rejection Requires an Additional Step that is Not Taught by any of the Cited References 16

5. The Examiner has Based the Rejection on an Alleged Absence in the Prior Art of a Motivation Not to Combine the References, Rather than a Clear Demonstration of a Motivation to Combine the References 17

C. Ground 2 of the Rejection: Kim in View of Wang and Saeki 18

1. Summary of the Examiner's Basis for Ground 2 of the Obviousness Rejection 18

2. Ground 2 of the Rejection is Improper for At Least the Same Reasons that Ground 1 of the Rejection is Improper 19

D. Conclusion 20

VIII. Claims Appendix 21

IX. Evidence Appendix 24

X. Related Proceedings Appendix 25

I. Real Party In Interest

The real party in interest in this appeal is The General Hospital Corporation.

II. Related Appeals and Interferences

There are no prior or pending appeals, interferences or judicial proceedings known to Appellant or the Appellant's legal representative which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in the present Appeal.

III. Status of Claims

Claims 1-39 and 46 have been canceled.

Claims 40-45 and 47-57 are rejected.

IV. Status of Amendments

No amendments have been filed subsequent to the Final Office Action dated April 3, 2006.

V. Summary of Claimed Subject Matter

A. Overview of Claimed Subject Matter

The invention defined by the claims on appeal relates generally to methods for producing herpes simplex virus (HSV) based-amplicons that contain a genomic DNA

insert. HSV-based amplicons are nucleic acid vectors derived from a herpes simplex virus. HSV-based amplicons can be packaged into infectious particles which can deliver foreign nucleic acids into cells with high efficiency. HSV-based amplicons can be used, *e.g.*, in gene therapy applications in which a foreign gene is delivered to one or more cells in a patient for therapeutic purposes.

HSV-based amplicons comprise a herpesvirus cleavage/packaging sequence (*pac*) and a herpesvirus origin of replication (*ori*). The *ori* element facilitates the replication of the amplicon DNA. The *pac* element allows the amplicon DNA to be packaged into infectious particles.

HSV-based amplicons provide a very efficient way of delivering foreign nucleic acids into cells via infection. Prior to the present invention, however, HSV-based amplicons were typically used to deliver only cDNAs into cells. Unlike *genomic DNA*, cDNAs are typically smaller nucleic acid molecules that lack the native regulatory regions (*e.g.*, native promoter) and introns that control the expression of genes in their normal context. The use of traditional HSV-based amplicons to deliver genomic DNA into cells is limited by the transgene capacity of HSV-based amplicons.

Large capacity cloning vectors have been developed that are capable of delivering very large genomic DNA inserts into cells. Exemplary large capacity cloning vectors include bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), P1 phage-based vector (PACs), cosmids and viral based vectors. Collections of large capacity cloning vectors, such as BACs, are available that contain overlapping genomic DNA fragments that cover the entire human genome. Large capacity cloning

vectors, however, do not contain a selection system or reporter genes suitable for expression in eukaryotic cells and cannot deliver foreign nucleic acids into cells by *infection*.

The vectors produced by the currently claimed methods combine the benefits of HSV-based amplicons (*e.g.*, highly efficient delivery of foreign nucleic acids into cells via infection) with the benefits of large capacity cloning vectors (*e.g.*, large genomic insert capacity and availability of constructs covering the entire human genome). In particular, the claims on appeal provide methods for converting a large capacity cloning vector into an HSV-based amplicon.

B. The Subject Matter Defined by Independent Claim 40 and Support in the Specification Therefor

The sole independent claim involved in this appeal is claim 40, which is directed to a method for converting a large capacity cloning vector into a herpes simplex virus (HSV)-based amplicon. The method comprises recombining: (a) a large capacity cloning vector comprising a genomic DNA insert; and (b) an amplicon vector comprising a herpesvirus cleavage/packaging sequence and a herpesvirus origin of replication; thereby producing an HSV-based amplicon vector comprising the genomic DNA insert. The claim further specifies that the large capacity cloning vector is a bacterial artificial chromosome (BAC), P1 phage-based vector (PAC), cosmid, yeast artificial chromosome (YAC), or viral based vector. Support for independent claim 40 can be found throughout the specification, for example, at page 12, paragraphs 0030 and 0032, and at page 24, paragraph 0104.

VI. Grounds of Rejection to be Reviewed on Appeal

There are two separate grounds of rejection to be reviewed on Appeal:

(1) ***First Rejection Under 35 U.S.C. § 103***: Whether the subject matter of claims 40-45 and 47-56 is obvious over Kim *et al.*, *Genome Res. 8*:404-412 (1998) ("Kim") (Exhibit 1) in view of Wang *et al.*, *J. Virol. 70*:8422-8430 (1996) ("Wang") (Exhibit 2) as evidenced by Woodfield *et al.*, *Nucl. Acids Res. 28*:3323-3331 (2000) ("Woodfield") (Exhibit 3).

(2) ***Second Rejection Under 35 U.S.C. § 103***: Whether the subject matter of claims 40, 56 and 57 is obvious over Kim in view of Wang and further in view of Saeki *et al.*, *Hum. Gene Ther. 9*:2787-2794 (1998) ("Saeki") (Exhibit 4).

VII. Argument

A. Legal Principles Relating to Nonobviousness

In order to establish a *prima facie* case of obviousness, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. *See In re Rouffet,* 149 F.3d 1350, 1357, 47 U.S.P.Q.2d 1453, 1457-58 (Fed. Cir. 1998). In addition, all of the claim limitations must be taught or suggested by the prior art. *See In re Royka*, 490 F.2d 981, 180 U.S.P.Q. 580 (CCPA 1974). Moreover, if an independent claim is nonobvious under 35 U.S.C. § 103, then

any claim depending therefrom is likewise nonobvious. *Se In re Fine*, 837 F.2d 1071, 5 U.S.P.Q.2d 1596 (Fed. Cir. 1988).

B. Ground 1 of the Rejection: Kim in View of Wang and Woodfield

1. Summary of Cited References

(a) Kim (Exhibit 1)

Kim refers to recombining a BAC (comprising a human genomic insert) with a reporter construct called "pRETRObac." (*See* Kim, page 406, Figure 1). pRETRObac contains a green fluorescent protein (GFP) reporter gene and a neomycin phosphotransferase (*neo*) selectable marker gene. (*See id.*) The purpose of the recombination reaction illustrated in Kim is to modify the BAC so that it can be selected for and identified in eukaryotic cells. (*See* Kim, page 404, right column, through page 405, top left column). There is no suggestion in Kim that the BAC could or should be modified in any way so that it can be delivered to cells by infection. Kim certainly provides no suggestion to use an amplicon vector in place of the pRETRObac construct.

(b) Wang (Exhibit 2)

Wang refers to a hybrid miniviral vector, "pH300-lac," that contains an HSV-1 origin of DNA replication, an HSV-1 viral packaging sequence, and the *LacZ* gene. (*See* Wang, page 8424, bottom left column, Fig. 1B). According to Wang, "The packaged pH300-lac DNA was very efficient in infecting human cells in tissue culture." (*See* Wang, page 8422, Abstract, lines 5-6). Wang, however, provides no suggestion to modify pH300-lac, or its parent vector pH300 in any way.

(c) Woodfield (Exhibit 3)

Woodfield is a supporting reference cited by the Examiner solely to support the proposition that "the recombination catalyzed by CRE recombinase also involves ligation of the recombined nucleic acids according to claim 53." (*See* August 9, 2005 Office Action, page 11, lines 9-11, citing the first sentence of the introduction of Woodfield).

2. Summary of the Examiner's Basis for the Rejection

According to the Examiner:

> It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the method of retrofitting a BAC clone of Kim *et al.* to insert the HSV-amplicon vector taught by Wang *et al.* to produce an HSV-based amplicon vector comprising said genomic DNA insert according to the method of the instant claims.

(*See* August 9, 2005 Office Action, page 10, lines 1-4). The Examiner further asserted that motivation to combine Kim and Wang comes from:

- The nature of the problem to be solved by the method of Kim, "which is to provide BAC clones comprising genomic DNA inserts with the capacity to transform sufficient numbers of mammalian cells . . . for functional analysis of the cloned inserts;"
- The alleged inefficiency of transfection using BAC vectors as used in Kim, "which necessitated time consuming and expensive antibiotic selection of transformed cells;" and
- The "very high gene transfer efficiency of the amplicon vector of Wang *et al.*"

(*See* August 9, 2005 Office Action, page 10, lines 5-12). As explained below, the Examiner's reasoning with regard to the alleged motivation to combine references is logically flawed and is not supported by the evidence.

3. A Person of Ordinary Skill in the Art Would Not Have Been Motivated to Modify and/or Combine the Cited References

(a) Kim Does Not Suggest a Need to Improve The Efficiency With Which Genomic DNA is Introduced Into Cells

The Examiner's basis for the rejection assumes that a person of ordinary skill in the art would have been motivated to improve the efficiency with which the BAC of Kim is introduced into cells. (*See, e.g.*, August 9, 2006 Office Action, page 10, lines 8-9). This is an incorrect assumption. There is nothing in Kim to suggest that the efficiency with which the retrofitted BAC is introduced into cells could or should be improved. In fact, considered as a whole, Kim suggests that the system set forth therein more than adequately allows for the introduction of genomic DNA into sufficient numbers of cells for functional analysis.

For instance, it is noted in Kim that "[w]e have described a simple and efficient method to modify BAC clones to contain additional genes in the BAC vector." (*See* Kim, page 408, sentence bridging left and right columns). It is further noted that "[e]ukaryotic cells that are transfected with a BAC that has been retrofitted with RETRObac are easily identified by FACS analysis or antibiotic selection. These modifications allow for the use of the retrofitted BAC clones as shuttle vectors and increase their utility in functional studies." (*See* Kim, page 409, top left column). In addition, Figures 3 and 4 on page 409 of Kim show the results of an experiment in which

a retrofitted BAC clone expressing GFP (clone "222N15") was transfected into human cells. According to Kim:

> Figure 3 shows the cells 1 week after transfection, viewed using either bright-field or fluorescent microscopy. Visual analysis after 2 days showed that ~10% of the cells were green. FACS analysis was performed to quantitate relative green fluorescence. Figure 4 presents a histogram showing relative fluorescence of SW480 cells transfected with parental BAC DNA or retrofitted BAC DNA. Whereas the parental transfection showed only background levels of green fluorescence (0.13%), cells transfected with retrofitted BAC DNA showed 5.73% of the cell population emitting green fluorescence.

(*See* Kim, page 407, right column).

Furthermore, Figure 5 on page 409 of Kim shows the results of an experiment in which murine NIH-3T3 cells were transfected with a retrofitted BAC containing human *p53* genomic DNA. RT-PCR analysis of the transfected cells confirmed expression of the genomic insert. (*See* Kim, paragraph bridging pages 407-408). Kim concludes by noting that:

> The use of modified BACs opens new areas for functional and biological research using genomic DNA. The large size of the genomic DNA insert in the BAC vector makes it likely that a single BAC will contain a gene in its entirety, along with its regulatory regions, and makes it an appealing target for introduction into cell lines to study gene expression, function, or regulation. Recent advances using RecA-assisted restriction endonuclease cleavage of large insert clones allos for site-specific mutagenesis and also deletions and fusions of BAC clones (Boren et al. 1996). These advances, in combination with BAC retrofitting, will permit researchers to examine the biological effects of wild-type genomic DNA contained on BAC clones, as well as mutant constructs generated by these methods.

(Kim, page 410, bottom left column).

When Kim is considered in its entirety, a person of ordinary skill in the art would have concluded that the system set forth in Kim more than adequately provides BAC clones comprising genomic DNA inserts having the capacity to transform sufficient numbers of mammalian cells for functional analysis of the cloned inserts. Kim does not indicate a need to improve the efficiency with which the modified BAC is introduced into cells. Thus, there would have been no reason for a skilled person to seek to modify or improve the system of Kim in any way and certainly would not have been motivated to use an amplicon vector in the system.

In response to the foregoing argument, the Examiner simply stated that:

> There is no requirement that a technology disclosed in a reference be wholly inoperative for its intended purpose or that an author or inventor explicitly state dissatisfaction with the functioning of their technology in order for the skilled artisan to be motivated to improve the technology. What is required for motivation is that the skilled artisan perceives some advantage or beneficial result of combining the teachings of the art.

(April 3, 2006 Office Action, page 4, lines 16-21). To support this statement, the Examiner cited MPEP § 2144. This section states that:

> The strongest rationale for combining references is a *recognition*, expressly or impliedly in the prior art or drawn from a convincing line of reasoning based on established scientific principles or legal precedent, that some advantage or expected beneficial result would have been produced by their combination. In re Sernaker, 702 F.2d 989, 994-95, 217 USPQ 1, 5-6 (Fed. Cir. 1983).

(M.P.E.P. § 2144, emphasis added). The Examiner has, however, failed to appreciate an essential element for establishing a motivation to combine references based on an

"expectation of some advantage," namely a *recognition* of the advantage. This recognition, according to the MPEP, can be: (a) expressed or implied in the prior art, or (b) drawn from a convincing line of reasoning based on established scientific principles or legal precedent.

The Examiner has not pointed to an express or implied recognition in the cited references of an advantage of recombining the BAC of Kim with the amplicon vector of Wang[1]. The asserted advantage of combining Kim with Wang, as set forth by the Examiner, is "to obtain a large number of mammalian cells comprising BAC clones without the need for antibiotic selection." (April 3, 2006 Office Action, page 4, lines 23-24). Nowhere in the cited references, however, is it recognized that this advantage could be realized by recombining an amplicon vector with a BAC. In fact, as explained in Section VII.B(3)(b), below, Wang indicates that efficient delivery of a DNA insert to cells can be achieved using the pH300-lac amplicon. Thus, a person of ordinary skill in the art, in view of the cited references, would not have recognized any advantage of recombining an amplicon vector with a BAC since the asserted "advantage" was already achieved by Wang using an amplicon vector *alone*. Thus, the principle set out in MPEP § 2144 and relied upon by the Examiner does not in any way support the rejection.

The Examiner has placed a great deal of emphasis on the statement in Kim that "[t]ransfection efficiencies of the modified BACs into human or murine cell lines ranged from 1% to 6%" as well as the results of Table 2 of Wang which shows the percentage of cells expressing LacZ ("X-gal positive cells") after being infected with pH300-lac. (*See*,

[1] Furthermore, the Examiner has not cited to "a line of reasoning based on established scientific principles or legal precedent" that demonstrates a recognition of the asserted advantage.

e.g., April 3, 2006 Office Action, page 5, line 16, through page 6, line 5). Since the *infection* efficiencies observed by Wang using an amplicon were reportedly higher than the *transfection* efficiencies observed by Kim using a BAC, the Examiner concluded that a person of ordinary skill in the art "would therefore perceive an expected benefit in substituting the amplicon vector." (*See* April 3, 2006 Office Action, page 6, lines 2-5).

What the Examiner has failed to appreciate, however, is the tremendous difference in the size of the constructs used in these two studies. The pH300-lac amplicon vector of Wang was only 18.2 kb. (*See* Wang, page 8424, Fig. 1B). The insert alone in the retrofitted BAC clones of Kim was approximately 140 kb. (*See* Kim, page 406, Figure 1). A person of ordinary skill in the art would have understood that the relatively lower transfection efficiencies mentioned in Kim were likely caused by the very large size of the BAC clones used in this reference as compared to the much smaller amplicon vector used in Wang.

Even if a skilled person were to ignore the difference in the size of the two constructs, the difference between the *transfection* efficiencies reported in Kim and the *infection* efficiencies reported in Wang would, at best, suggest that infection using an amplicon is more efficient than transfection using a BAC. There is no indication in the cited references that the infection efficiencies observed with the amplicon of Wang could somehow be conferred upon the BAC of Kim. In other words, the Examiner has set forth an "apples versus oranges" comparison that would not in any way suggest a combination of the two references. Even if a person of ordinary skill in the art were to appreciate that the amplicon of Wang was delivered to cells with greater efficiency than the retrofitted BAC clone of Kim, the skilled person would still not have had any reason

or motivation to combine these two references and, more particularly, would not have had any reason to *recombine* an amplicon with a BAC.

(b) The Examiner's Asserted Advantage of Combining the References Could Have Been Achieved Most Directly Using the Vector of Wang Without Any Modification or Combination

The Examiner's asserted rationale in support of the rejection, even if correct, does not provide any motivation for one of ordinary skill in the art to combine the reference teachings. According to the Examiner, "[w]hen the teachings of Kim *et al.* and Wang *et al.* are viewed as a whole, the skilled artisan would clearly perceive an advantage in using the amplicon vector of Wang *et al.*" (April 3, 2006 Office Action, page 4, lines 21-23). If this assertion is correct, it logically follows that the skilled person would simply use the amplicon vector of Wang to deliver genomic DNA into cells without having to use a BAC at all. According to Wang:

> A miniviral vector [*i.e.*, amplicon] combines the advantages of cloning the transgene in bacteria and virus-mediated high efficiency of gene transfer. The theoretical capacity for large insertion into such a vector offers the possibility to carry large DNA fragments including regulatory elements.

(Wang, page 8422, bottom left column). Thus, Wang clearly indicates that very efficient delivery of large genomic DNA inserts to cells can be achieved simply by cloning the genomic insert into the amplicon vector of Wang using the same standard molecular biological techniques illustrated in this reference. For Example, Wang notes that "pH300-lac was constructed by inserting a *lacZ* gene into the *Hin*dIII and *Not*I sites of the multiple cloning site of pH300." The Examiner has not presented any explanation as to why a person of ordinary skill in the art would have been motivated to recombine a

BAC containing a genomic DNA insert with the amplicon vector of Wang, when the genomic DNA insert itself could have simply been inserted into the multiple cloning site of the Wang vector using standard restriction enzyme and ligation reactions -- which is the method specifically illustrated by Wang.

Stated differently, the result that the Examiner asserts a person of ordinary skill in the art would have been motivated to achieve -- *i.e.*, improving the efficiency of genomic DNA delivery into cells without the use of antibiotic selection -- could have been achieved by simply cloning the genomic insert into the amplicon vector. There is nothing in either Kim or Wang that would have suggested *recombining* the entire BAC of Kim with the amplicon of Wang.

In response to the above argument, the Examiner stated that "standard restriction enzyme and ligation reactions are within the broadest reasonable construction of the claims in light of the specification." (July 13, 2006 Advisory Action, continuation sheet 1, lines 26-27). Thus, the Examiner has construed the term "recombining," as recited in the present claims, as encompassing "standard restriction enzyme and ligation reactions." Regardless of whether the Examiner's claim construction is correct, the claims specify recombining "*a large capacity cloning vector* comprising a genomic DNA insert" and an amplicon vector. Thus, cloning a genomic DNA fragment directly into the vector of Wang using standard restriction enzyme and ligation reactions (which, in light of the teachings of Wang, is the most straightforward way to create a vector for the infectious delivery of a genomic DNA insert) is outside of the scope of the present claims in any event.

The Examiner also asserted in the Advisory Action that:

> [R]ecombining the entire BAC with the amplicon is suggested by the teachings of Kim et al., which concern converting BACs comprising human genomic DNA to vectors suitable for transformation of mammalian cells by retrofitting BAC vectors with elements that enable their propagation in mammalian cells.

(July 13, 2006 Advisory Action, continuation page 1, lines 28-31, emphasis in original). This statement is logically flawed and does not support the rejection. As noted above, Kim simply refers to a method by which selectable markers and reporter genes can be inserted into BACs using the pRETRObac plasmid. Nowhere in Kim is it suggested that an amplicon could or should be used in place of the pRETRObac construct (which is simply used as a vehicle for inserting GFP and *neo* genes into a BAC clone). (*See* Kim, page 406, Figure 1). The Examiner has failed to explain how or why a person of ordinary skill in the art would possibly consider replacing the pRETRObac construct of Kim with the amplicon vector of Wang.

Finally, in response to Applicants' assertion that the Examiner's asserted advantage of combining the references could have been achieved using the amplicon vector of Wang without any need to recombine it with a BAC containing a genomic DNA insert, the Examiner stated that:

> Kim et al. is the primary reference used in the rejection and is relied upon to teach the method. Wang et al. is relied upon only to teach the elements of an HSV-based amplicon and the efficient delivery of genes using HSV-based amplicons.

(July 13, 2006 Advisory Action, continuation page 1, lines 32-35). Thus, according to the Examiner, the Wang reference -- aside from its disclosure of HSV-based amplicons and the use of HSV-based amplicons to deliver genes -- should somehow be ignored simply because Wang is not the "primary reference" relied upon by the Examiner to justify the rejection. Regardless of which reference the Examiner considers the "primary reference," however, a person of ordinary skill in the art would have had knowledge of the entirety of *both* references. Thus, in view of the method illustrated in Wang (in which a DNA insert is ligated into the multiple cloning site of pH300), a person of ordinary skill in the art would appreciate that the efficient delivery of genomic DNA to cells could have been achieved by directly subcloning a genomic DNA insert into the amplicon vector of Wang -- a process that would fall outside of the scope of the present claims. A skilled person would have seen no reason whatsoever, in the light of the cited references, to recombine the amplicon vector of Wang with the BAC of Kim.

The Examiner's above-quoted statement (regarding the use of Kim as the "primary reference" for the rejection) clearly indicates that the Examiner, in making the obviousness rejection, has simply reconstructed the currently claimed methods by picking and choosing different elements from the cited references without pointing to any clear motivation to make such a combination. In order to manufacture a "motivation" to combine the references, the Examiner has chosen to ignore the entirety of the Wang reference which clearly suggests that genomic DNA inserts can be delivered to cells simply by cloning the genomic DNA insert directly into an amplicon vector by standard restriction enzyme and ligation techniques. Instead of considering Wang for all it discloses, the Examiner has focused on the Kim reference and has filled in the gaps by

selectively citing Wang "only to teach the elements of an HSV-based amplicon and the efficient delivery of genes using HSV-based amplicons." This type of hindsight reconstruction is legally improper. "One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention." *In re Fine*, 837 F.2d 1071, 1075, 5 U.S.P.Q.2d 1596, 1599-1600 (Fed. Cir. 1988).

4. The Examiner's Basis for the Rejection Requires an Additional Step that is Not Taught by any of the Cited References

The Examiner has acknowledged that an additional step that is neither taught nor suggested by the cited references would be required in order to arrive at a method that falls within the scope of the currently presented claims. For example, the Examiner stated that:

> The teachings of Kim et al., and the generally high level of skill in the art, provide all that is necessary to retrofit a DNA a BAC clone by site-specific recombination, including *inserting a recombination site into an HSV amplicon* to be used in retrofitting a BAC clone according to the method of Kim et al.

(July 13, 2006 Advisory Action, continuation page 2, lines 30-33, emphasis added). Thus, the Examiner seems to appreciate that, in order to concoct a method that falls within the scope of the present claims using Kim and Wang as the starting point, a person of ordinary skill in the art would first have needed to "insert a recombination site into an HSV amplicon." Significantly, the Examiner has not even alleged that any of the cited references teach or suggest this additional step. Absent some teaching or suggestion in the cited references of inserting a recombination site into an HSV amplicon, the Examiner's basis for the rejection cannot be maintained.

5. The Examiner has Based the Rejection on an Alleged Absence in the Prior Art of a Motivation Not to Combine the References, Rather than a Clear Demonstration of a Motivation to Combine the References

As noted in the MPEP, "[t]he examiner bears the initial burden of factually supporting any prima facie conclusion of obviousness. . . . The initial burden is on the examiner to provide some suggestion of the desirability of doing what the inventor has done." *See* M.P.E.P. § 2142. As explained above, the Examiner's asserted "motivation" to combine Kim and Wang amounts to nothing more than impermissible hindsight reconstruction of the claimed methods and requires at least one additional step that is neither taught nor suggested by the cited references. In addition, the Examiner has improperly based the rejection on an alleged absence in the prior art of a motivation *not to combine* the references. In particular, the Examiner asserted that:

> There is nothing of record to indicate that the skilled artisan *would not have been motivated* to use the method of retrofitting BAC vectors as taught by Kim et al. and there is clearly no teaching in the art of record to suggest that the skilled artisan *should exclude* the BAC vector when producing an amplicon as suggested by the cited art.

(July 13, 2006 Advisory Action, continuation page 1, lines 35-38, emphases added). Thus, the Examiner, in making the rejection, has emphasized what *is not* taught in the references rather than what *is* taught in the references.

Since the Examiner has the initial burden of providing clear and particular evidence of a motivation to combine references, an assertion that "there is nothing of record to indicate that the skilled artisan would not have been motivated" to combine the references cannot form the basis of a *prima facie* case of obviousness. Likewise, a *prima facie* case of obviousness cannot be established based on an alleged absence in the prior

art of a suggestion to exclude the teachings of one of the references. Instead, to establish a *prima facie* case of obviousness, the Examiner must point to "clear and particular" evidence of a motivation to combine the references. *See In re Dembiczak*, 175 F.3d 994, 999, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999). The Examiner's burden cannot be met simply by pointing to what the evidence does *not* teach.

C. Ground 2 of the Rejection: Kim in View of Wang and Saeki

1. Summary of the Examiner's Basis for Ground 2 of the Obviousness Rejection

The rejection of claims 40, 56 and 57 under § 103(a) based on Kim in view of Wang and Saeki (Ground 2 of the rejection) assumes that the subject matter of claims 40 and 56 would have been obvious in view of Kim and Wang (Ground 1 of the rejection). According to the Examiner:

> Claims 40 and 56 are obvious over the teachings of Kim *et al.* in view of Wang *et al.* for the reasons set forth herein above.
>
> Claim 57 is directed to the method of claim 56 wherein the packaging is accomplished using a helper virus-free system.
>
> * * *
>
> It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the method of Kim *et al.* in view of Wang *et al.* to include packaging of the HSV-based amplicon using a helper virus-free system as taught by Saeki *et al.* Motivation to combine the teachings is found in Wang *et al.*, who identifies the possibility that a helper virus-free packaging system might be developed for HSV-based amplicons as among the advantages of the vectors. Motivation also comes from Saeki *et al.*, who identifies helper-virus contamination as a source of toxicity in HSV-based amplicon preparations. In view of these teachings, the

> skilled artisan would clearly be motivated to use a helper virus-free system, such as that described by Saeki *et al.*, in a method comprising packaging an HSV-based amplicon vector as recited in the instant claims. Absent evidence to the contrary, one would have a reasonable expectation of success in combining these teachings in view of the teachings of Saeki *et al.*, which demonstrate the efficiency of the helper virus-free system described therein.

(August 9, 2005 Office Action, page 12, line 15, through page 13, line 4).

2. Ground 2 of the Rejection is Improper for At Least the Same Reasons that Ground 1 of the Rejection is Improper

As explained in detail in section VII.B, above, the rejection of claims 40-45 and 47-56 as being unpatentable over Kim in view of Wang and Woodfield is improper because:

(1) A person of ordinary skill in the art would not have been motivated to modify and/or combine Kim and Wang, especially since there is no indication in the cited references of a need to improve the transfection efficiency that was achieved using the system of Kim;

(2) If a person of ordinary skill in the art wished to achieve the advantages asserted by the Examiner (*i.e.*, efficient genomic DNA delivery to cells without antibiotic selection), this could have been accomplished most straightforwardly by following the techniques of Wang, which involve simply cloning the DNA insert into an amplicon vector using standard restriction enzyme and ligation techniques. Such a method would fall outside the scope of the currently presented claims;

(3) In order to arrive at a method that falls within the scope of the claims on appeal, the modification/combination of the references suggested by the Examiner admittedly requires an additional step, *i.e.*, the insertion of a recombination site into an HSV-based amplicon vector, which is neither taught nor suggested by the cited references; and

(4) The Examiner's rationale for the rejection at best relies upon an assertion of what is not taught in the cited references, rather than a demonstration of clear and particular evidence of a motivation to combine the references.

For at least these same reasons, the rejection of claims 40, 56 and 57 based on Kim, Wang and Saeki is likewise improper.

D. Conclusion

Since the Examiner has not established a *prima facie* case of obviousness under either Ground 1 or Ground 2 of the rejection, Appellants respectfully request that the Board reverse all outstanding rejections and remand this application to issue.

Respectfully submitted,

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VIII. Claims Appendix

40. A method for converting a large capacity cloning vector into a herpes simplex virus (HSV)-based amplicon, said method comprising recombining:

(a) a large capacity cloning vector comprising a genomic DNA insert; and

(b) an amplicon vector comprising a herpesvirus cleavage/packaging sequence and a herpesvirus origin of replication;

thereby producing an HSV-based amplicon vector comprising said genomic DNA insert; wherein said large capacity cloning vector is a bacterial artificial chromosome (BAC), P1 phage-based vector (PAC), cosmid, yeast artificial chromosome (YAC), or viral based vector.

41. The method of claim 40, wherein said herpesvirus cleavage/packaging sequence is an HSV-1 cleavage/packaging sequence.

42. The method of claim 40, wherein said herpesvirus origin of replication is an HSV-1 origin of replication.

43. The method of claim 40, wherein said herpesvirus cleavage/packaging sequence is an HSV-1 cleavage/packaging sequence, and said herpesvirus origin of replication is an HSV-1 origin of replication.

44. The method of claim 40, wherein said amplicon vector of (b) further comprises a genetic element from Epstein-Barr virus (EBV).

45. The method of claim 44, wherein said genetic element from EBV is *oriP*.

47. The method of claim 40, wherein said large capacity cloning vector is a bacterial artificial chromosome (BAC).

48. The method of claim 40, wherein said large capacity cloning vector is a P1 phage-based vector (PAC).

49. The method of claim 40, wherein said recombining comprises site-specific recombination of (a) and (b) in the presence of a site-specific recombinase.

50. The method of claim 49, wherein said site-specific recombinase is selected from the group consisting of: P1 bacteriophage CRE, yeast FLP, and yeast R recombinase.

51. The method of claim 49, wherein said site-specific recombinase is P1 bacteriophage CRE.

52. The method of claim 40, wherein said recombining comprises homologous recombination of (a) and (b).

53. The method of claim 40, wherein said recombining comprises ligation of (a) and (b).

54. The method of claim 40, wherein said genomic DNA insert is 50 to 100 kb in size.

55. The method of claim 40, wherein said genomic DNA insert is 110 to 150 kb in size.

56. The method of claim 40, further comprising packaging said HSV-based amplicon vector comprising said genomic DNA insert into an infectious particle.

57. The method of claim 56, wherein said packaging is accomplished using a helper virus-free system.

IX. Evidence Appendix

Exhibit	Title of Exhibit	Location in Record
1	Kim *et al.*, *Genome Res. 8*:404-412 (1998)	Cited by the Examiner in the Office Action issued on August 9, 2005.
2	Wang *et al.*, *J. Virol. 70*:8422-8430 (1996)	Cited by the Examiner in the Office Action issued on August 9, 2005.
3	Woodfield *et al.*, *Nucl. Acids Res. 28*:3323-3331 (2000)	Cited by the Examiner in the Office Action issued on August 9, 2005.
4	Saeki *et al.*, *Hum. Gene Ther. 9*:2787-2794 (1998) ("Saeki")	Cited by the Examiner in the Office Action issued on August 9, 2005.

X. Related Proceedings Appendix

No decisions have been rendered by a court or the Board in any related proceeding.

GENOME METHODS

Modification of Bacterial Artificial Chromosome Clones Using Cre Recombinase: Introduction of Selectable Markers for Expression in Eukaryotic Cells

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Bacterial artificial chromosome clones (BACs) are widely used at present in human genome physical mapping projects. To extend the utility of these clones for functional genomic studies, we have devised a method to modify BACs using Cre recombinase to introduce a gene cassette into the *loxP* sequence, which is present in the vector portion of the BAC clone. Cre-mediated integration is site specific and thus maintains the integrity of the genomic insert sequences, while eliminating the steps that are involved in restriction digest-based DNA cloning strategies. The success of this method depends on the use of a DNA construct, RETRObac, which contains the reporter marker green fluorescent protein (GFP) and the selectable marker neomycin phosphotransferase (*neo*), but does not contain a bacterial origin of replication. BAC clones have been modified successfully using this method and the genomic insert shows no signs of deletions or rearrangements. Transfection efficiencies of the modified BACs into human or murine cell lines ranged from 1% to 6%. After culture in media containing G418 for 3 weeks, ~0.1% of cells previously sorted for GFP expression acquired stable antibiotic resistance. Introduction of a human BAC clone that contains genomic *p53* sequences into murine NIH3T3 cells led to expression of human *p53* mRNA as determined by RT-PCR, demonstrating that sequences contained on the BAC are expressed. We believe that GFP–*neo* modified BAC clones will be a valuable resource in efforts to study biological effects of known genes as well as in efforts to clone and analyze new genes and regulatory regions.

The recent effort to construct high resolution physical maps of the human genome have made use of genomic libraries contained in several cloning vectors, including yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), human artificial episomal chromosomes (HAECs), P1 artificial chromosomes (PACs), P1 vectors (P1s), and cosmid clones (Deaven et al. 1986; Burke et al. 1987; Ioannou et al. 1994; Shepherd et al. 1994; Sun et al. 1994; Kim et al. 1996). BAC libraries are widely used at present because they are commercially available and they have the advantage of containing large inserts that average 140 kb (Shizuya et al. 1992). In addition, BACs are very stable because they are based on the *Escherichia coli* F-factor plasmid, which maintains a very low copy number in bacterial cells, thus minimizing the possibility of recombination and resultant chimeric clones.

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In spite of these advantages, BACs cannot be used as shuttle vectors because they do not contain a selection system or reporter genes suitable for expression in eukaryotic cell lines. Thus, when BACs of interest are identified, it is necessary to modify or "retrofit" them to use them for biological studies. Current methods of retrofitting BACs rely on performing restriction enzyme digests to isolate genomic fragments, followed by ligation into vectors that contain the desired markers (Mejia and Monaco 1997) or on homologous recombination with a shuttle vector that must be constructed specifically, based on the sequences present in the genomic insert (Yang et al. 1997). Newer BAC libraries, based on modified vectors that contain genes that can be expressed in mammalian cell lines have been devised; however, these clones do not correlate with those of existing libraries (Baker and Cotten 1997). Thus, a method whereby existing BAC clones could be modified easily to contain specific marker genes

8:404–412 ©1998 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/98 $5.00; www.genome.org

would be highly desirable for their subsequent use in biological assays.

Here, we report a method to retrofit BAC clones using site-specific recombination between *loxP* sites contained on the BACs and on a modifying construct. The *loxP* sequence is derived from bacteriophage P1 and consists of two 13-bp inverted repeats separated by an 8-bp spacer (Sternberg and Hamilton 1981; Abremski et al. 1983; Fukushige and Sauer 1992). Bacteriophage P1 also encodes Cre recombinase, which catalyzes site-specific recombination between *loxP* sites. Recombination between *loxP* sites that are carried on separate vectors results in integrative recombination. The success of this method is based on the use of a plasmid-based construct, RETRObac, which carries the modifying genes, but does not contain a bacterial origin of replication, and thus cannot replicate within a bacterial host cell unless it has been integrated into the BAC clone. The use of RETRObac minimizes background and obviates the need to screen resultant clones to determine whether the modification has been successful. This strategy provides the following advantages: the insert genomic DNA remains undisturbed, it does not require knowledge of the DNA sequence of the insert, it is independent of restriction sites present in the insert, and it provides the flexibility to introduce any markers of interest into the BAC clone.

We have used this method to introduce the genes for green fluorescent protein (GFP) and neomycin phosphotransferase (*neo*) into BAC clones. No rearrangements or deletions were detected in the modified BACs. Several modified clones were introduced into mammalian cell lines, from which long-term stable integrants were derived that expressed genomic sequences contained within the BAC clone. We believe that this retrofitting strategy using RETRObac will prove to be a valuable resource for isolating and analyzing new genes, as it allows for direct selection of cells that contain transfected BACs. The large size of the genomic insert in the BAC vector improves the chances that a single BAC clone will contain a gene in its entirety, along with its regulatory regions, making them well suited for eukaryotic genetic transfer studies.

RESULTS

Preparation of the Plasmid pBGLS and the Retrofitting Construct RETRObac

Plasmid pEGFP-C1 contains GFP under control of the constitutive CMV promoter. GFP is an intrinsically fluorescent jellyfish protein that requires no cofactors (Chalfie et al. 1994; Cheng et al. 1996; Levy et al. 1996). When introduced into mammalian cells, expression of GFP can be monitored by means of fluorescent microscopy or fluorescence-activated cell sorter (FACS) analysis. The plasmid also contains *neo* under control of a bacterial β-lactamase promoter and a viral SV40 promoter, thus conferring resistance to kanamycin in bacteria and G418 in mammalian cells. The following elements were introduced into pEGFP-C1: a β-lactamase promoter, a *loxP* site, and a *lacZ* gene. In addition, *AscI* restriction enzyme sites were placed on both sides of the pUC origin of replication, resulting in the plasmid pBGLS (Fig. 1). An *AscI* restriction digest of pBGLS results in a 7.5-kb fragment and a 1-kb fragment, which contains the pUC origin of replication. Gel purification of the 7.5-kb fragment and its subsequent ligation produced the retrofitting construct RETRObac.

Retrofitting BAC Clones With RETRObac

Cre recombinase was used to catalyze in vitro recombination between *loxP* sites contained on the BAC clone and RETRObac. Figure 1 shows the retrofitting scheme and the resultant modified BAC clone. Recombination of RETRObac with a BAC clone results in a modified BAC, which has dual antibiotic resistance, chloramphenicol (from the BAC vector) and kanamycin (from RETRObac). Recombination at the *loxP* sites also separates the *lacZ* gene from its promoter. This allows for color screening of clones that have recombined at the *loxP* sites, as nonrecombined species are blue when grown on plates containing X-gal, whereas recombined clones are white.

Trial experiments using pBGLS as the retrofitting construct showed that virtually all of the colonies that were resistant to both chloramphenicol and kanamycin were blue (396 of 397 colonies in one trial experiment). This suggested that these colonies contained both the plasmid and an unmodified BAC clone, or that they contained a modified BAC clone as well as additional copies of the high copy number plasmid. Because both of these events would display blue staining, it would have been necessary to screen these colonies further to identify those that contained the modified BAC clones. To avoid this time consuming procedure, we decided to remove the pUC origin of replication from pBGLS. Because RETRObac cannot replicate within a host cell unless it has integrated into the BAC clone, all bacterial colonies selected for dual

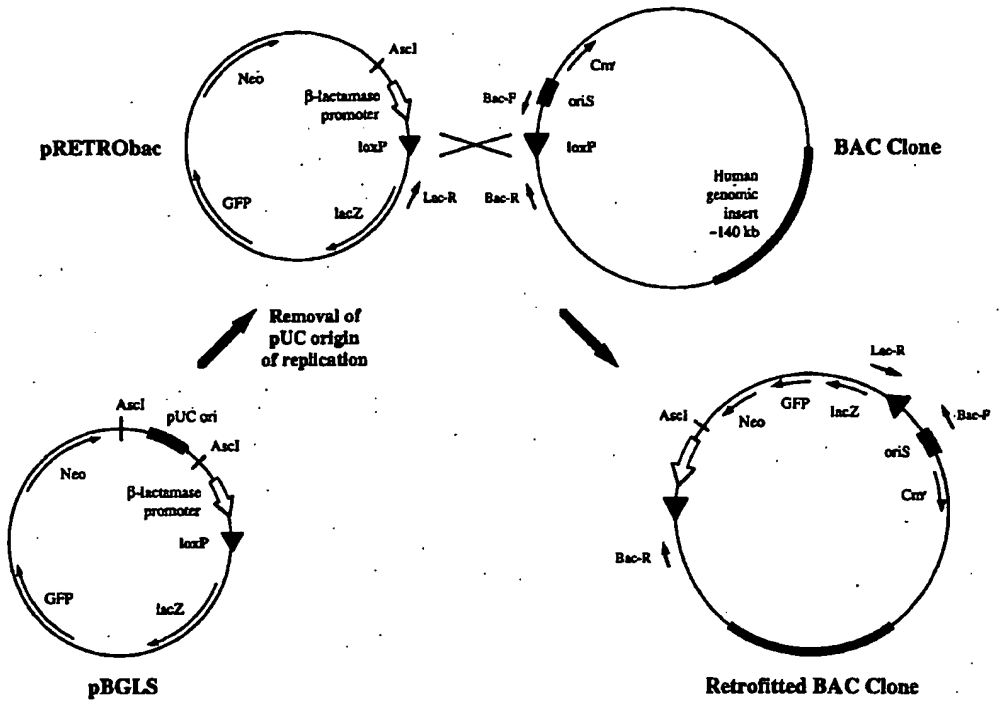

Figure 1 Preparation of RETRObac and a schematic diagram illustrating BAC retrofitting. The plasmid pBGLS contains the genes GFP, *neo,* and *lacZ*. *Asd* digestion of pBGLS followed by gel purification of the 7.5-kb fragment and its subsequent ligation results in the final retrofitting construct RETRObac, which lacks the bacterial origin of replication. Recombination between RETRObac and a BAC clone at the *loxP* sites leads to integration of all of RETRObac into the BAC clone, leaving the genomic insert unaltered. The approximate positions of PCR primers Bac-F, Bac-R, and Lac-R are indicated. Constructs are not drawn to scale.

antibiotic resistance should contain retrofitted BAC clones only.

Table 1 shows the results of a retrofitting experiment using BAC clone 261M17 and RETRObac in the presence of X-gal. Recombination resulted in colonies that were resistant to both chloramphenicol and kanamycin only when Cre recombinase was added. In addition, all of these colonies were white, suggesting that recombination had occurred at the *loxP* site, thus separating the *lacZ* gene from its promoter. The inability to obtain any colonies that were resistant to both antibiotics in the absence of Cre shows that recombination is completely dependent on the presence of the recombinase. The number of colonies that were resistant to both antibiotics, compared to chloramphenicol-resistant only ($230/10^6$ colonies), allowed an estimate of the efficiency of Cre-mediated recombination at ~0.024%. When colonies were selected with kanamycin only, roughly the same number of colonies were obtained as compared to selection with both antibiotics. However, a small fraction of these colonies were blue, most likely representing colonies that contained pBGLS plasmid, which had escaped in vitro removal of pUC origin of replication sequences. We have obtained comparable results for all seven BAC clones that have been modi-

Table 1. Results of Retrofitting BAC Clone 261M17

	Colonies (no.)			
	Cre^+		Cre^-	
Antibiotic selection	(white)	(blue)	(white)	(blue)
Chloramphenicol	960,000	0	184,000	0
Kanamycin	280	20	0	10
Chloramphenicol and kanamycin	230	0	0	0

fied by this method (033B22, 067L19, 149K11, 222N15, 228I10, 261M17, 350A20).

PCR analysis allows for the identification of clones that have recombined specifically at the *loxP* site. Whereas the primer pair Bac-F and Bac-R detects a 238-bp product in the parental nonrecombined BAC clone, primer pair Bac-F and Lac-R detects a 181-bp product only if recombination has taken place at the *loxP* site. Figure 2A shows the results of PCR analysis of 16 randomly selected chloramphenicol–kanamycin-resistant colonies after retrofitting of BAC clone 261M17. Parental BAC clones were positive only for a 238 bp product. In contrast, 15 of 16 retrofitted clones were positive for the 181-bp product, suggesting that for the vast majority of clones, recombination had occurred specifically at the *loxP* site. Note that PCR across a *loxP* site generally results in a doublet, which we attribute to the secondary structure of this sequence that contains two 13-bp inverted repeat sequences.

To determine whether any deletions of the genomic insert in the retrofitted BAC clones had occurred, we perfomed pulsed field gel electrophoresis (PFGE) after an *Asc*I restriction digest. Because RETRObac was engineered to contain an *Asc*I site, retrofitting results in an additional *Asc*I site in modified BAC clones. Figure 2B shows the results of PFGE of colonies obtained from the retrofitting of BAC clone 261M17. Of a total of 19 chloramphenicol–kanamycin-resistant colonies, eight randomly selected clones were digested with *Asc*I. All eight clones showed a single band measuring 100 kb, whereas the parental BAC clone was uncut by *Asc*I, suggesting that gross deletions of the modified DNA had not occurred in any of the clones that were tested. For all seven of the BACs that have been retrofitted, two colonies were analyzed using PFGE. In all cases, PFGE after an *Asc*I digest showed that the size of the resultant band (or bands) in the modified clones was ~7 kb larger than the parental band, consistent with integration of RETRObac, which measures 7.5 kb. A *Not*I restriction digest resulted in two additional fragments in the modified clones of 4 and 3.5 kb, which were too small to resolve by PFGE but were seen when separated using conventional electrophoresis.

To detect whether rearrangement of the genomic DNA had occurred during recombination, we used DNA fingerprint analysis after a *Hin*dIII restriction digest. Figure 2C shows the results of one of these experiments, analyzing 10 of 19 randomly selected colonies after the retrofitting of BAC clone 261M17. All 10 retrofitted clones showed an identical banding pattern, suggesting that rearrangement of the DNA had not occurred. The parental banding pattern showed three differences as compared to the retrofitted banding pattern, as expected because of integration of RETRObac.

Transfection and Expression of Retrofitted BAC Clones in Mammalian Cell Lines

Liposome-mediated transfection was used to introduce DNA from retrofitted BAC clones into the SW480 human colorectal cancer cell line. Preliminary experiments using plasmid pEGFP-C1 to introduce the reporter gene GFP showed a transfection efficiency of 25%, as determined by FACS analysis. The same conditions were used to transfect 10 μg of DNA from retrofitted BAC clone 222N15. Figure 3 shows the cells 1 week after transfection, viewed using either bright-field or fluorescent microscopy. Visual analysis after 2 days showed that ~10% of the cells were green. FACS analysis was performed to quantitate relative green fluorescence. Figure 4 presents a histogram showing relative fluorescence of SW480 cells transfected with parental BAC DNA or retrofitted BAC DNA. Whereas the parental transfection showed only background levels of green fluorescence (0.13%), cells transfected with retrofitted BAC DNA showed 5.73% of the cell population emitting green fluorescence. GFP-positive cells (10^4) were collected and cultured in media containing G418 for 3 weeks. Stable antibiotic resistance was obtained in 0.1% of these cells, half of which also retained their fluorescence, verifying that retrofitted BAC clones express their marker genes.

To determine whether retrofitted BAC clones express genes contained within their genomic sequences, human BAC clone 261M17, which contains *p53* genomic sequences, was transfected into the murine NIH-3T3 cell line, which expresses wild-type murine *p53*. Two days after transfection, pooled cells were analyzed by RT–PCR to detect *p53* expression. In several experiments, we were unable to detect message using human-specific p53 primers, although murine-specific p53 primers always resulted in a positive signal. These results suggested that either human genomic *p53* was not being expressed or that we were not detecting its expression, possibly because of low level expression of the human gene in the heterologous system. To differentiate between these possibilities we selected for long-term integrated clones by adding the antibiotic G418 to the culture media for 4 weeks. Control transfections using DNA from parental BAC clones did not result in any colonies during this period.

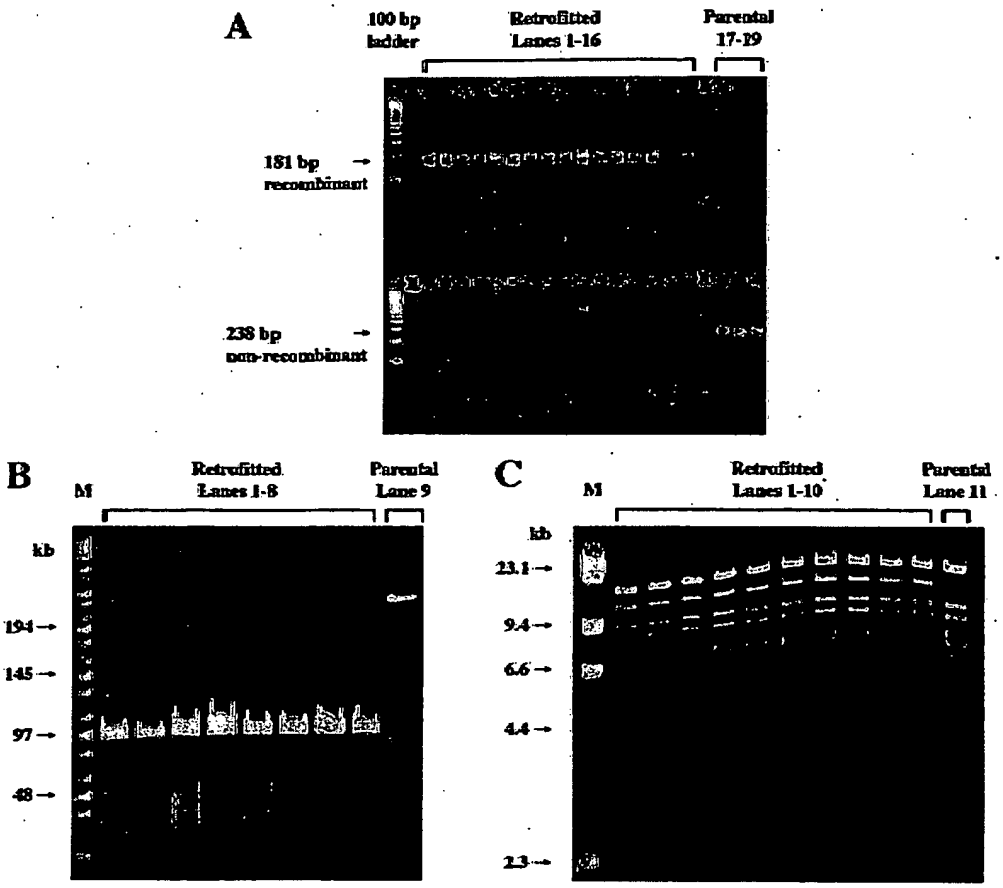

Figure 2 Analysis of randomly selected colonies obtained after recombination of BAC clone 261M17 and RETRObac. (*A*) Whole cell PCR of retrofitted clones (lanes *1–16*) and parental BAC clones (lanes *17–19*). (*Top*) Of 16 clones, 15 are positive for an expected 181-bp recombined product using primers Bac-F and Lac-R, whereas all parental clones are negative. (*Bottom*) Only the parental clones are positive for a 238-bp nonrecombined product using primers Bac-F and Bac-R. (X) Skipped lanes. (*B*) PFGE after *Asc*I restriction digest of retrofitted clones (lanes *1–8*) or the parental BAC clone (lane *9*). All eight retrofitted clones show a single band of ~100 kb, whereas the parental clone is undigested by *Asc*I. (*C*) Fingerprint analysis after *Hin*dIII restriction digest of retrofitted clones (lanes *1–10*) or the parental BAC clone (lane *11*). All 10 retrofitted clones show identical bands, whereas the parental clone has three differences.

Cells were then pooled and subjected to RT–PCR analysis. As seen in Figure 5, all cells express endogenous murine *p53* mRNA. However, only cells transfected with retrofitted P53 BAC DNA were positive for human *p53* message. As expected, a control transfection using DNA from retrofitted BAC clone 67L19 was negative for human *p53* expression.

DISCUSSION

We have described a simple and efficient method to modify BAC clones to contain additional genes in the BAC vector. Specifically, the method was used to introduce a gene cassette, RETRObac, which contains the reporter marker GFP and the selectable marker *neo*, into any BAC clone. Combining purified RETRObac and BAC DNA along with Cre recombinase in vitro leads to integrative recombination between the *loxP* sites contained on both constructs. We refer to this procedure as BAC retrofitting, indicating a retroactive modification to a construct, a term first coined to describe targeted integration of the yeast-selectable marker *LYS2* and the mammalian-selectable marker thymidine kinase into a YAC clone by homologous recombination

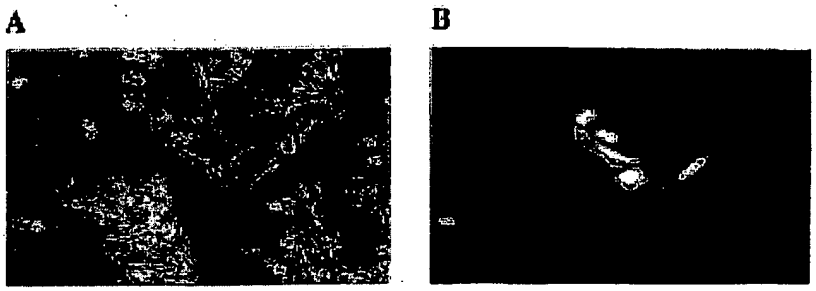

Figure 3 SW480 cells, 7 days after transfection using DNA from retrofitted BAC clone 222N15. (*A*) A bright-field view of the cells at 200× magnification. (*B*) The same field of cells viewed using fluorescent microscopy.

(Eliceiri et al. 1991). Subsequent retrofitting strategies used Tn*10*-mediated insertion of *neo* into P1 clones (Sternberg 1994). Eukaryotic cells that are transfected with a BAC that has been retrofitted with RETRObac are easily identified by FACS analysis or antibiotic selection. These modifications allow for the use of the retrofitted BAC clones as shuttle vectors and increase their utility in functional studies.

The success of this method rests in large part on the use of the retrofitting construct RETRObac, which contains a *loxP* site but does not contain a bacterial origin of replication. This modification minimizes the background that results from bacterial colonies that are resistant to both chloramphenicol and kanamycin and that contain the BAC clone and pBGLS plasmid. With RETRObac replacing pBGLS, all colonies with dual antibiotic resistance contain retrofitted BACs. Additional screening of recombinant clones by monitoring for expression of β-galactosidase is possible, but not necessary.

RETRObac is applicable to the widely used BAC libraries provided by Research Genetics (Huntsville, AL) and Genome Research (St. Louis, MO). A similar approach can be used to retrofit any vector, provided it contains a *loxP* site and a suitable antibiotic resistance gene. The retrofitting of existing PAC and P1 clones would require modification of RETRObac to replace kanamycin with an alternative antibiotic resistance gene, such as ampicillin, as these clones are already resistant to kanamycin.

Existing BAC clones are generally unsuitable for eukaryotic expression studies and BAC libraries based on newer vectors have been constructed to make them more useful for this purpose (Baker and

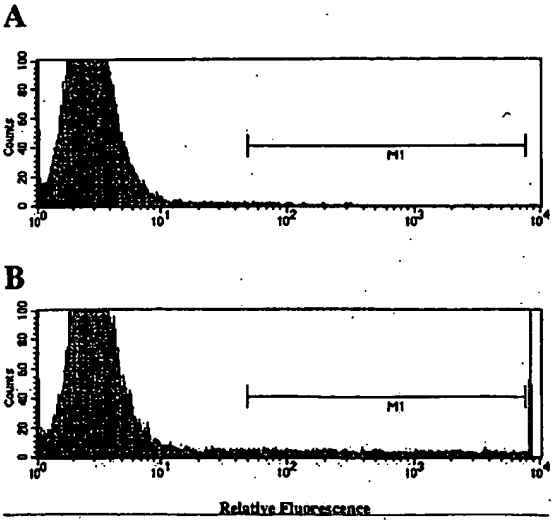

Figure 4 FACS analysis of GFP expression in transfected SW480 cells performed 2 days after transfection of modified or unmodified BAC DNA. (*A*) Profile of cells transfected with DNA from parental nonretrofitted BAC clone 222N15 shows low-level background fluorescence (0.13%) of gated viable cells. (*B*) Cells transfected with DNA from the retrofitted BAC clone shows a population emitting green fluorescence (5.73%) using markers from 1.4 to 3.7 log fluorescence.

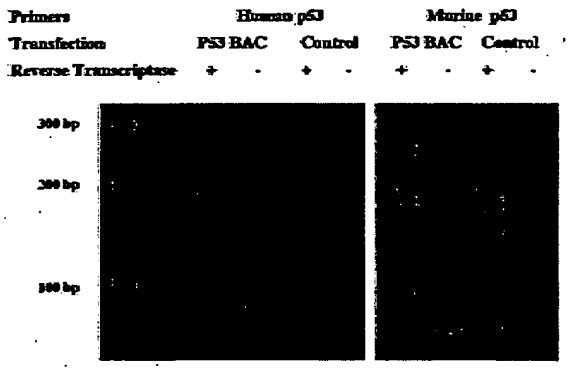

Figure 5 RT–PCR analysis of NIH-3T3 cells transfected with retrofitted BAC DNA. Cells were transfected with DNA from retrofitted BAC clone 261M17, which contains *p53* genomic sequences (P53 BAC) or BAC clone 67L19 (Control), cultured for 4 weeks in the presence of G418 and pooled for analysis. RT–PCR using primers specific for murine *p53* (*right*) was positive for an expected 194-bp product in both transfections. RT–PCR using primers specific for human *p53* (*left*) was positive for an expected 199-bp product only in cells that were transfected with P53 BAC DNA. All lanes were negative when reverse transcriptase was not added.

Cotten 1997). However, it is not possible to correlate clones from these libraries with existing clones. Other investigators have devised methods to modify existing BAC clones. Mejia and Monaco (1997) describe modification of BACs and PACs by isolating *Not*I-digested insert DNA and then ligating it into a vector containing a *lacZ* reporter gene and *neo*. This traditional DNA cloning approach is straightforward and has been used successfully to modify BAC clones. Yang et al. (1997) describe the retrofitting of BAC clones in *E. coli* using homologous recombination of a shuttle vector that has been designed specifically to integrate into a known sequence in the genomic DNA. Pronuclear injection of one modified BAC into fertilized mouse zygotes resulted in transgenic mice that transmitted the intact BAC in their germ line.

The retrofitting method that we describe provides several advantages over these previous approaches. Because our procedure relies on a well-characterized site-specific bacteriophage recombination system, it is independent of the restriction sites present in the genomic DNA, which remains unaltered. The same vector can be used for all BACs and does not need to be adapted to the specific genomic sequences necessary for homologous recombination. Dual antibiotic selection and an optional color screen guarantees that the vast majority of resultant clones will have been successfully retrofitted. Because of this, it is theoretically possible to perform all of the modification and selection steps in microtiter plates, without individual verification of clones. Thus, the method might be applied potentially to modify a gridded BAC library, while maintaining the grid coordinate reference system.

The use of modified BACs opens new areas for functional and biological research using genomic DNA. The large size of the genomic DNA insert in the BAC vector makes it likely that a single BAC will contain a gene in its entirety, along with its regulatory regions, and makes it an appealing target for introduction into cell lines to study gene expression, function, or regulation. Recent advances using RecA-assisted restriction endonuclease cleavage of large insert clones allows for site-specific mutagenesis and also deletions and fusions of BAC clones (Boren et al. 1996). These advances, in combination with BAC retrofitting, will permit researchers to examine the biological effects of wild-type genomic DNA contained on BAC clones, as well as mutant constructs generated by these methods. In addition, because the BAC vector also contains a *cosN* site, the modified BAC clone can be linearized with the enzyme λ-terminase. Linearization of the BAC may improve chromosomal integration and genetic fidelity of the integrated clones (Rackwitz et al. 1984), although it might decrease the expression of vector genes from unintegrated DNA, thus possibly precluding their use in direct FACS selection. We believe that our retrofitting procedure will prove to be a valuable resource for identifying and analyzing new genes as it allows for functional biological studies using both dominant selection and flow cytometry analysis.

METHODS

Isolation of BAC Clones and BAC DNA

DNA pools of a human BAC library were obtained from Research Genetics. Individual BAC clones were isolated in two rounds of 48 PCR reactions using various published primers (Bookstein et al. 1994) or primers that we designed using sequences obtained from GenBank (accession nos. M81104, X01237, X54156, X00884, X00885). The clone numbers reported in this paper correspond to the designation of Research Genetics. DNA was prepared using Qiagen tip-500 columns (Chatsworth, CA) and the suggested protocol modified for BACs as follows. Cells were inoculated in 1 liter of LB media containing 25 μg/ml chloramphenicol and incubated overnight at 37°C. The culture was divided into two 500 ml preparations, centrifuged, resuspended in buffer P1 containing 1 mg/ml lysozyme, and incubated at room temperature for 15 min. DNA was eluted from tip-500 columns by adding three 5-ml aliquots of buffer QF, which was heated to 65°C. Typical yields for BAC DNA ranged from 25 to 60 μg.

Preparation of the Retrofitting Construct RETRObac

Plasmid pEGFP-C1, which contains GFP and *neo*, was obtained from Clontech (Palo Alto, CA). PCR primers LoxP-F (aagtttacagtacatatgactagtacaatggaagtccgagctcat) and LoxP-R (ataagtattaatcatatgacgcgtctctgatgccgcatagttaa) were used to amplify a 156-bp portion of the BAC clone, containing the *loxP* sequence and a *Not*I restriction enzyme site. The primers contain a *Spe*I restriction site at one end and flanking *Nde*I sites. Restriction digest of the amplified fragment with *Nde*I and pEGFP-C1 with *Ase*I results in 4-bp compatible overhangs, which were ligated to each other. The *lacZ* gene was isolated as a *Not*I fragment from the plasmid pCMVβ (Clontech) and inserted into the *Not*I site of the amplified fragment. Complementary oligonucleotides promoter-F (ctagggcgcgcctctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttt) and promoter-R (ctagaaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaggcgcgcc), which contain the minimal 35 bp of the bacterial β-lactamase promoter as well as an *Asc*I restriction site were synthesized, annealed to each other, and cloned into the *Spe*I site to drive expression of the *lacZ* gene. Finally, the resulting plasmid was digested with *Bsa*I, the overhangs filled in using T4 DNA polymerase and an *Asc*I linker (New England BioLabs, Beverly, MA) was inserted, resulting in the plasmid pBGLS. To prepare the construct RETRObac, pBGLS was digested with *Asc*I, resulting in a 7.5-kb fragment and a 1-kb fragment that contains the pUC origin of replication. The 7.5-kb fragment was purified from a 1% agarose gel using

a QIAquick gel extraction kit (Qiagen), ligated, and the DNA purified using a QIAquick PCR purification kit (Qiagen) following the suggested protocols, resulting in the final BAC retrofitting construct RETRObac.

Retrofitting of BAC Clones

Cre enzyme was obtained from Novagen (Madison, WI), and the suggested protocol was used with the following modifications: 500 ng of BAC DNA, 50 ng of RETRObac, 1 unit of Cre recombinase, and 1× reaction buffer were added to an Eppendorf tube in a total volume of 30 µl. After incubations for 60 min at 37°C, 5 min at 70°C, 10 min at room temperature, 60 min on ice, phenol–chloroform–isoamyl extraction and ethanol precipitation were performed. The DNA was resuspended in 15 µl of H_2O and added to 20 µl of DH10B electrocompetent cells. The mixture was transferred to a chilled 0.1-cm electroporation cuvette and electroporated at 1.8 kV, 200 Ω, 25 µF using a Bio-Rad Gene Pulser (Hercules, CA) (Sheng et al. 1995). One milliliter of LB media was added and incubated for 1 hr at 37°C, and aliquots were plated on LB plates containing 25 µg/ml chloramphenicol and 25 µg/ml kanamycin, prespread with 50 µl of 2% X-gal.

PCR and PFGE Analysis of Retrofitted Clones

A forward primer in the BAC clone (Bac-F, aggaaacgacaggtgctgaa) and reverse primers in the BAC clone (Bac-R, atatggtgcactctcagtacaatctg) and also in the *lacZ* fragment of pRETRObac (Lac-R, gctttagcaggctctttcgatc) were designed. Primer pair Bac-F and Bac-R amplifies a 238-bp product from the native BAC vector, whereas primer pair Bac-F and Lac-R amplifies a 181-bp product when *lacZ* has integrated. Whole-cell PCR amplification was performed by growing individual bacterial colonies overnight and using 2 µl of cells in 20 µl of total PCR reaction. PFGE was performed using a CHEF-DR II electrophoresis unit (Bio-Rad). Restriction-digested DNA (325 ng) was separated on a 1% agarose gel using a pulse of 5–25 sec at 180 V for 20 hr.

FACS Analysis of SW480 Cells

The cell line SW480 was obtained from ATCC (Rockville, MD) and grown in a 37°C 10% CO_2 humidified incubator in Dulbecco's modified Eagle media supplemented with 4 mM L-glutamine, 10 mM HEPES, penicillin–streptomycin, and 10% fetal bovine serum. Cells (10^6) were plated into 100-ml tissue culture plates and incubated overnight. The following day, cells at 50% confluence were transfected using 60 µg of lipofectin (Life Technologies, Gaithersburg, MD) and 10 µg of retrofitted BAC DNA following the suggested protocol. Mock-transfected cells were prepared similarly, but no DNA was added to the lipofectin. Serum-supplemented medium was added 6 hr after transfection. The cells were viewed 48 hr after transfection using an Olympus inverted fluorescence microscope and then resuspended in phosphate-buffered saline containing 1 µg/ml propidium iodide for FACS analysis. Mock transfected cells were used to obtain background levels of fluorescence, and gates were used to exclude both debris and dead cells. Cells were analyzed and sorted on a Becton-Dickinson FACS Vantage (San Jose, CA). A 200 mW argon laser emitting at 488 nm was used to excite the cells and the fluorescence emission was detected in a bandpass filter of 530/30. GFP-positive cells (10^4) were collected and grown in media containing 1.5 mg/ml G418 for 3 weeks.

RT-PCR Analysis of NIH-3T3 Cells

NIH-3T3 cells were obtained from ATCC and treated similarly to SW480 cells with the following exceptions. Cells were grown in DMEM with 10% calf serum and 5×10^5 cells were plated into six-well tissue culture plates. Transfection was performed using 20 µg of lipofectamine (Life Technologies) and 4 µg of retrofitted BAC DNA. Serum supplemented medium was added 6 hr after transfection, and medium containing 1.0 mg/ml G418 was added 48 hr later and selection continued for 4 weeks. Total RNA from pooled clones was prepared using Trizol reagent (Life Technologies) following the suggested protocol. cDNA preparation and PCR were performed in one reaction using Titan RT-PCR reagent (Boehringer Mannheim, Indianapolis, IN) using an annealing temperature of 55°C and the products separated on a 4% Nusieve gel (FMC BioProducts, Rockland, ME). Primer pairs for RT-PCR were designed from exons 7 (P53h7-F, cctcaccatcatcacactgg) and 9 (P53h9-R, ctggggagaggagctggtgttgtt) to amplify a 199-bp human *p53* product and from exons 10 (P53m10-F, tgctacagaggagtctggagac) and 11 (P53m11-R, gtgtctcagccctgaagtcataa) for a 194-bp murine *p53* product.

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A Hybrid Herpesvirus Infectious Vector Based on Epstein-Barr Virus and Herpes Simplex Virus Type 1 for Gene Transfer into Human Cells In Vitro and In Vivo

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We have developed a miniviral vector, pH300, based on the human herpesviruses 1 and 4, herpes simplex virus type 1 (HSV-1), and Epstein-Barr virus (EBV), carrying EBV sequences for plasmid episomal maintenance and HSV-1 sequences for amplification and packaging in multimeric form into HSV-1 capsids in the presence of a helper virus and helper cell line. A reporter gene, the bacterial *lacZ* gene, which expressed β-galactosidase, was inserted into the multiple cloning site of pH300 to make pH300-lac. The packaged pH300-lac DNA was very efficient in infecting human cells in tissue culture. The pH300-lac miniviral stock was used to infect in vitro various human cell types derived from breast cancer, lung cancer, and liver cancer. Up to 95% of cells were infected and expressed β-galactosidase activity after exposure to viral stock at a multiplicity of infection of 3. There was essentially no apparent cytotoxicity after infection of cultured cells in vitro. To test in vivo gene delivery, human liver tumor cells preimplanted subcutaneously in nude mice and injected *in situ* with pH300-lac showed high efficiency of ectopic gene expression. The pH300 miniviral vector is a simple and effective gene transfer system which shows potential for gene therapy of cancer and inherited diseases.

Gene therapy has been considered a revolutionary method for treating genetic diseases, cancer, and infectious diseases, such as AIDS. The method of transfer of foreign genetic material into cultured human cells and/or the human body will play a fundamental role in the gene therapy practice. As current vectors, such as retroviruses, adenoviruses, and vaccinia viruses, are tested in clinical trials, their disadvantages and limitations are becoming apparent (1, 12, 17, 19, 33). Manipulation of an entire virus as a gene transfer vector generally involves recombination of the gene of interest into the viral genome. Such recombinant viruses are rather difficult to handle, and their capacity for insertion of exogenous DNA is relatively limited, from 7.5 kb (adenovirus) to 30 kb (herpes simplex virus type 1 [HSV-1]). Although HSV-1 has a relatively larger insert capacity, current vectors appear cytotoxic as a result of residual viral proteins produced by the virus (11). Helper virus-dependent miniviral vectors whose capacity for insertion is theoretically as large as the size of the original viral genome are being developed (31, 33). These vectors carry *cis*-acting viral elements required for replication and packaging into infectious virions. Since these vectors are defective for viral production, they are dependent on a helper virus to provide the missing viral proteins in trans. A miniviral vector combines the advantages of cloning the transgene in bacteria and virus-mediated high efficiency of gene transfer. The theoretical capacity for large insertion into such a vector offers the possibility to carry large DNA fragments including regulatory genomic elements. Importantly, a miniviral vector can be designed for the desired mode of action by assembling several elements from different viruses, therefore creating a hybrid miniviral vector system. Finally, a helper virus-free packaging system similar to that of other viral vectors (5, 18, 23) could be developed for an HSV-1 amplicon (7a).

The linear double-stranded DNA genome of HSV-1 is 152 kb in length and encodes at least 72 unique proteins (16). The viral genome contains three origins of replication, one within the unique long segment (oriL) and two within the repeats flanking the unique short segment (oriS) (25, 27). An HSV-1 vector has a number of advantages as a gene delivery system. These include a wide host range, the ability to infect nonreplicating cells like neurons in which the vectors can be maintained indefinitely in a latent state (26), and the ability to prepare high-titer viral stocks. By using a plasmid containing an HSV lytic origin of replication and HSV terminal packaging signal sequences, Frenkel et al. (15, 24, 25, 32) demonstrated that such an amplicon was amplified and packaged into infectious HSV virions in the presence of a wild-type helper virus. The virions contained multimeric forms of the original monomeric vector conforming to a rolling-circle replication mode. Since the wild-type helper virus invariably caused cell death due to lytic replication in infected cells, two replication-defective HSV systems have been developed as helper viruses. In a helper virus temperature-sensitive system, the virion stocks were produced at the permissive temperature (31°C). Infection of cells at 37°C allowed miniviral vector delivery to the target cell, whereas the temperature-sensitive mutant helper virus was incapable of entering the lytic cycle and thereby cell death was prevented (8, 9, 28). In another system, an essential immediate-early gene 3 (IE3), encoding the ICP4 protein necessary for early and late viral gene expression and virus replication, had been deleted from the helper viral genome. The miniviral vector DNA was transfected into a helper cell line which expressed a functional IE3 gene for complementation and virion propagation was induced by infecting cells with the IE3 deletion mutant replication-incompetent helper virus (6, 9).

Epstein-Barr virus (EBV) is another member of the human

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herpesvirus family. EBV carries a unique latent replication origin (oriP) which allows viral self-replication in human cells without entering the lytic cycle (14, 21, 36). oriP-based vectors allow episomal replication and maintenance in cells expressing the EBV nuclear antigen EBNA-1, the only virus-encoded transactivator of oriP (37). EBV-based miniviral vectors for delivering large genes to human cells which can be stably maintained in an episomal form in these cells have been developed (3, 29–31). In this study, we present a novel EBV/HSV-based miniviral vector, pH300, allowing high-efficiency *lacZ* gene transfer into various human cells in vitro and in vivo. The vector contains the HSV lytic replication origin, oriS, and an HSV packaging sequence, a, which allow vector replication and packaging in the presence of the IE3 gene-deleted helper virus in an IE3 gene-expressing helper E5 cell line. The latent replication origin, oriP, and the transactivator, EBNA-1, from EBV allow vector episomal maintenance in the E5 cells so that viral stocks of high titers can be made. Effective β-galactosidase expression in infected human cells, particularly in various tumor-derived cell lines, is demonstrated. The efficiency of infection can be as high as 95 to 99% in cultured human fibroblast cells and epithelial cells without apparent cytotoxicity. As an in vivo model for gene transfer, packaged pH300-lac virions were injected into preimplanted human liver tumors in nude mice. Histological 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining showed *lacZ* gene expression in many areas of the injected tumor. The pH300 miniviral vector system should be a useful and efficient DNA delivery system for gene expression studies and gene therapy experiments.

MATERIALS AND METHODS

Construction of pH300 and pH300-lac. pH300 is a 15.6-kb plasmid which was constructed from a combination of several plasmids. The HSV replication origin, oriS, is a *Bam*HI fragment of pT011, kindly provided by N. Stow, Medical Research Council, Glasgow, United Kingdom). The HSV packaging sequence, a, is an *Eco*RI-*Hin*dIII fragment of pT011. The EBNA-1 gene and oriP of EBV, and the Hyg^r gene, which codes for hygromycin B phosphotransferase, are from a BamHI fragment of p500 (34). The backbone of the vector is pCDMV2A (kindly provided by T. Tsukada, Kyoto University, Kyoto, Japan), which includes a cytomegalovirus (CMV) promoter and simian virus 40 (SV40) poly(A) site, elements required for bacteria growth, as an expression cassette consisting of a *Sca*I-*Nru*I fragment from pCDM8 (INVITROGEN); a multiple cloning site and a 1.1-kb *Eco*RI-*Pst*I stuffer fragment are derived from pCH110 (LKB, Pharmacia). pH300-lac was constructed by inserting a *lacZ* gene into the *Hin*dIII and *Not*I sites of the multiple cloning site of pH300. The *lacZ* gene was a *Hin*dIII-*Not*I fragment from pCDMV3-lac kindly provided by H. Takebe, Kyoto University.

Cells and viruses. All cells were from the American Type Culture Collection except where indicated. Cells were grown and maintained in Eagle's minimal essential medium (E-MEM; Gibco BRL) (or in RPMI 1640 where indicated) containing 10% fetal bovine serum (HyClone), glutamine, and penicillin-streptomycin and incubated at 37°C in a humidified 5% CO_2 incubator. E5 is a helper cell line derived from African green monkey cells (Vero) and stably transfected with an IE3 gene of HSV (6) (kindly provided by S. Bachenheimer, University of North Carolina at Chapel Hill [UNC-CH]). The cells were maintained in 400 μg of G-418 (Geneticin; GibcoBRL) per ml. NHF cells are fibroblasts derived from normal skin (kindly provided by W. Kaufmann, UNC-CH). VA13 cells (American Type Culture Collection) were established from human W138 cells by SV40 transformation. XP4BE cells are SV40-transformed skin fibroblasts derived from a patient with xeroderma pigmentosum, group variant (an inherited DNA repair defect). JML cells were established from tumor cells of a patient with Li-Fraumeni syndrome. GM6914A cells are SV40-transformed skin fibroblasts derived from a patient with Fanconi's anemia. T98G cells are fibroblast-like human glioma cells. SKBR-3, MCF-7, T47D, and DT-20 are all human breast carcinoma cell lines. T47D and SKBR-3 cells were grown and maintained in RPMI 1640. RD cells are spindle-shaped human rhabdomyosarcoma cells (kindly provided by B. Weissman, UNC-CH). SW1271, A498, and HepG2 are human lung, kidney, and liver carcinoma cell lines, respectively. The HSV-1 strain 17$^+$ IE3 deletion mutant D30EBA (20) was kindly provided by P. Johnson, University of California, San Diego. The virus was grown and titers were determined in E5 cells.

Transfection and selection for hygromycin resistance. Transfection of pH300-lac into E5 cells was carried out with Lipofectin as recommended by the manufacturer (GIBCO BRL/Life Technologies, Gaithersburg, Md.). Optimal results were obtained by using 2 μg of plasmid DNA and 10 μg of Lipofectin, each diluted in 1 ml of Opti-MEM (GIBCO BRL/Life Technologies); the components were mixed well and incubated at room temperature for 15 min. The liposome-DNA complex was added to 5×10^5 actively growing E5 cells (washed previously with Opti-MEM) in 2 ml of Opti-MEM in a six-well plate (Falcon, Lincoln Park, N.J.). The Opti-MEM was replaced with complete medium after the cells were incubated for 12 to 15 h at 37°C in a humidified 5% CO_2 incubator. Two days after transfection, the cells were trypsinized and seeded into 10-cm-diameter dishes at 10^6 per dish. The medium was replaced the following day by fresh medium containing 200 μg of hygromycin B (ICN Biomedical, Inc., Aurora, Ohio) per ml to select stable cell transformants.

Packaging and preparation of virion stocks. Hygromycin-resistant colonies carrying the episomal pH300-lac were trypsinized, and 5×10^5 cells were plated onto a 35-mm-diameter dish. At cell confluency, helper virus in 0.5 ml of Opti-MEM was added to the dish at an MOI (multiplicity of infection) of 1. The viruses were allowed to adsorb to the cells for 3 h at 37°C in a humidified 5% CO_2 incubator. Viral solutions were then aspirated, and 3 ml of E-MEM with 10% of fetal bovine serum was added to the cells, which were kept for 3 days at 37°C in a humidified 5% CO_2 incubator. The medium was collected and centrifuged at $1,000 \times g$ for 10 min, and the supernatant was used for virus titration and infection. For production of helper virus, helper virus was used at an MOI of only 0.1.

Titration. (i) Helper virus. Viral stocks were diluted in 100 μl of Opti-MEM, and 10^{-3} to 10^{-8} dilutions were used to infect confluent monolayers of E5 cells grown in 24-well plates. The viruses were allowed to adsorb to the cells for 3 h at 37°C in a humidified 5% CO_2 incubator. The virus solutions were aspirated and overlaid with 1 ml of E-MEM containing 5% fetal bovine serum and 1% low-melting-point agarose which was previously equilibrated to 42°C. The low-melting-point agarose medium was allowed to solidify at room temperature and then placed at 37°C in a humidified 5% CO_2 incubator for 3 days. Plaques were then visualized by staining with 0.5 ml of 0.6% crystal violet in 50% ethanol for 5 min. The plates were dried, and plaques were counted. Titers of helper virus were expressed as PFU per milliliter.

(ii) Minivirus. pH300-lac miniviral vector stocks were titered by infection of T98G cells as described above. Assuming that one X-Gal-positive T98G cell represented one infectious pH300-lac virion, the titers were expressed as blue cell-forming units per milliliter.

In vitro and In vivo infection with pH300-lac. For in vitro infection, cultured human cells from different sources, including a number of tumor cell lines (see Table 2), were trypsinized, counted, and seeded at appropriate cell densities. When cells reached confluency, they were infected by defective viral pH300-lac at an MOI of 3 for 3 h. For in vivo studies, female nude mice (Harlan Laboratories) were injected subcutaneously at two symmetrically located sites with 10^7 HepG2 cells suspended in 0.1 ml of phosphate-buffered saline (PBS). When visible tumors approximately 0.5 cm^3 in size developed 1 month later, miniviral pH300-lac was administered to the tumor at one site by a multidirection injection in situ with 0.2 ml of viral solution, approximately 2×10^6 PFU. As a control, the tumor on the other side of the mouse was injected with 0.2 ml of PBS. The β-galactosidase activity was measured 24 h later for both in vitro and in vivo infection as described below.

Assays for β-galactosidase activity. Two assays were used to detect β-galactosidase activity in pH300-lac infected cells. For visualization of virions carrying the *lacZ* gene, infected cells were rinsed with PBS on day 2 following infection. The cells were then fixed for 5 min at room temperature in 2% formaldehyde–0.3% glutaraldehyde in PBS and stained by incubation in a chromophore solution containing 0.1% X-Gal (Promega), 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 5 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS. Incubation continued at 37°C until color developed (usually within 30 to 60 min). For quantitative measurement of β-galactosidase activity, all cells were trypsinized after 48 h of infection and washed once in PBS solution. Then 10^6 cells were resuspended in an *o*-nitrophenyl-β-D-galactopyranoside (ONPG) lysis solution (0.45 mM ONPG and 0.5% Nonidet P-40 in Hanks balanced salt solution) and incubated at 37°C in a 5% CO_2 humidified incubator, and the optical density at 420 nm was read 1 h later. Expression of β-galactosidase activity in tumor tissue was detected as described previously (4). Briefly, freshly isolated tissue was flash-frozen in isopentane and cooled in liquid nitrogen for the preparation of cryosections. These cryosections (~8 to 10 mm) were fixed briefly with glutaraldehyde and histochemically stained as described for cell monolayers but incubated for 4 to 16 h.

UV irradiation of virus. Stocks of packaged pH300-lac were irradiated with UVC light (254 nm) at various dosages. The effect of UV irradiation on *lacZ* gene expression of pH300-lac, i.e., the β-galactosidase activity, was determined by infection of T98G cells at an MOI of 3. X-Gal staining and measurement of ONPG expression levels were both carried out 24 h later.

Slot blot and DNA hybridization. DNA from packaged pH300-lac and helper virus (D30EBA) was prepared and transferred to a nylon membrane (Magnagraph Nylon; MSI) for slot blot hybridization analysis (2). One million copies of pH300-lac and pGH83, the plasmid carrying the HSV IE110 promoter (kindly provided by S. Bachenheimer), were also loaded as copy number standards. Membranes were hybridized with pH300-lac miniviral vector-specific (hygromycin, *Bgl*II-*Eco*RI fragment, 1,068 bp) and HSV helper virus-specific (IE110 promoter, *Hin*dIII-*Bam*HI fragment, 950 bp) probes. The radioactive signals were exposed and analyzed on a PhosphorImager (Molecular Dynamics). The data were corrected by the fact that the packaged multimeric pH300-lac virions

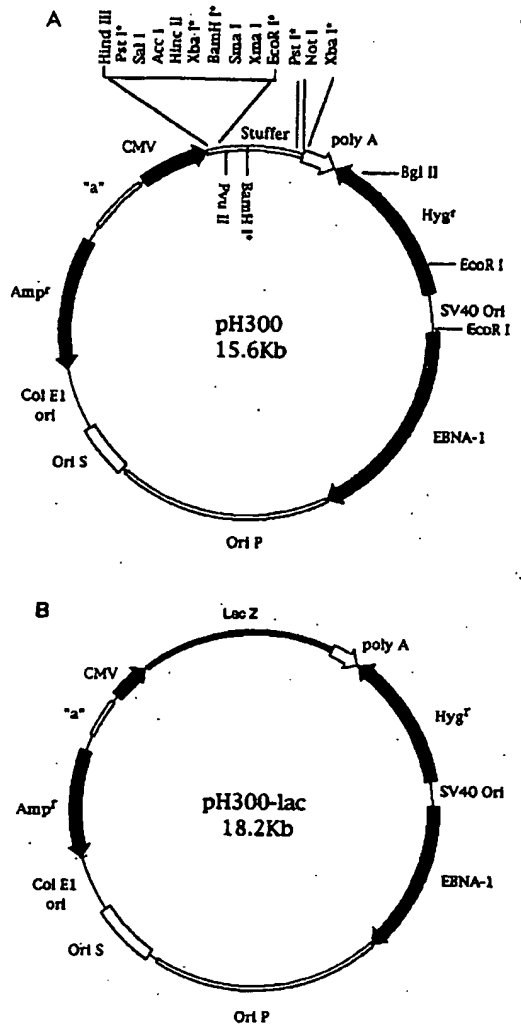

FIG. 1. Structures of pH300 and pH300-lac mini-EBV/HSV-1 vectors. pH300 and pH300-lac have the same structure. pH300 has a multiple cloning site and a stuffer fragment for multipurpose insertion and cloning. pH300-lac contains a constitutively expressed *lacZ* gene inserted at the *Hin*dIII and *Not*I sites of pH300. Ori S, HSV-1 origin of DNA replication. "a," HSV packaging sequence; Ori P, EBV origin of DNA replication; SV40 Ori, SV40 origin of DNA replication; CMV, CMV immediate-early promoter; poly A, SV40 early region poly(A) sequence for termination of transcription; Amp^r, ampicillin resistance gene; Col E1 ori, pBR322 replication origin.

contained about eight copies of pH300-lac plasmid whereas the helper virus contained only two copies of the HSV IE110 promoter.

RESULTS

Strategy for the generation of the hybrid EBV/HSV-1 pH300 and pH300-lac miniviral vectors. The 15.6-kb mini-HSV-1 vector, pH300, is schematically shown in Fig. 1A. pH300 contains the HSV-1 sequences oriS (HSV-1 origin of DNA replication) and a (viral packaging). oriP and EBNA-1 of EBV allow episomal maintenance of pH300 in human cells under hygromycin B selection. The SV40 origin has a bidirectional promoter function, with the early and late promoters driving the Hyg^r and EBNA-1 genes, respectively. The expression cassette consists of a CMV immediate-early promoter and the SV40 early region poly(A) sequence for termination of transcription to drive transgene expression. In addition, a multiple cloning site and a stuffer fragment are included for easy subcloning. The miniviral reporter vector pH300-lac (Fig. 1B) is essentially the same as pH300 except for the insertion of the *lacZ* gene between the *Hin*dIII and *Not*I sites of pH300.

TABLE 1. Efficiency of packaging pH300-lac DNA into HSV-1 particles

Viral stock[a]	pH300-lac titer[b]	D30EBA titer[c]	Ratio[d]
1	3.0×10^6	2.0×10^5	15
2	2.3×10^6	2.0×10^6	1.15
3	2.56×10^6	1.0×10^7	0.256
4	2.56×10^6	8.0×10^6	0.32
5	1.3×10^6	3.0×10^6	0.433
6	4.3×10^6	2.2×10^6	1.95
7	4.1×10^6	1.3×10^7	0.35
8	4.0×10^4	1.0×10^6	0.04
9		2×10^6	
10		4×10^6	
11		3.5×10^6	

[a] Viral stocks consist of a mixed population of pH300-lac and helper virus D30EBA (1 to 8) or helper virus only (9 to 11). Viral stocks 1 to 7 were produced after pH300-lac transfection into E5 cells and selection with hygromycin; viral stock 8 was produced immediately after transfection without hygromycin selection; viral stocks 9 to 11 were produced from original E5 cells.

[b] Titers of pH300-lac miniviral vector stocks were determined by infection of T98G cells as blue cell-forming units per milliliter.

[c] D30EBA (helper virus) titers were determined in E5 cells as PFU per milliliter.

[d] Ratios were obtained by dividing pH300-lac titer by D30EBA titer.

Production of packaged pH300-lac HSV-1 miniviral virions. pH300-lac was transfected into cultured E5 cells and subjected to selection with hygromycin B. After 2 to 3 weeks, stable resistant colonies were replated and infected with the helper virus D30EBA. The virion stocks consisted of a mixed population of miniviral pH300-lac and helper D30EBA. Titers of the produced stocks and ratios of pH300-lac to D30EBA are presented in Table 1. The packaging efficiency of pH300-lac was very reproducible in different preparations, ranging between 2.5×10^6 and 5×10^6 blue cell-forming units/ml. The production of helper virus, however, varied between different stocks, resulting in ratios of packaged pH300-lac to D30EBA ranging from 15 to 0.256. When a pH300-lac viral stock was prepared after transient transfection of pH300-lac into E5 cells without hygromycin selection and after infection with helper virus, less pH300-lac DNA was packaged and the ratio of pH300-lac to helper virus was only 0.04. Such a low ratio is similar to those observed by others by others with HSV amplicon vectors (13). To confirm the packaging of pH300-lac, slot blot hybridization was performed (Fig. 2). This analysis showed that the ratios of pH300-lac to D30EBA viral particles were in the range of 1.98 to 0.165.

Effective gene delivery by infectious pH300-lac into various human cell types. Cultured normal and genetically defective human cells as well as a number of tumor cells from various tissues (Table 2) were infected with miniviral pH300-lac, and β-galactosidase activity was evaluated by X-Gal staining. As illustrated in Fig. 3, NHF cells, normal primary human fibroblasts which are resistant to standard transfection methods, were very efficiently infected (Fig. 3A), as was JML, a tumor cell line derived from a patient with Li-Fraumeni syndrome (Fig. 3B). XP4BE and GM6914A, SV40-transformed human fibroblast lines derived from patients with the DNA repair defect xeroderma pigmentosum variant and Fanconi's anemia,

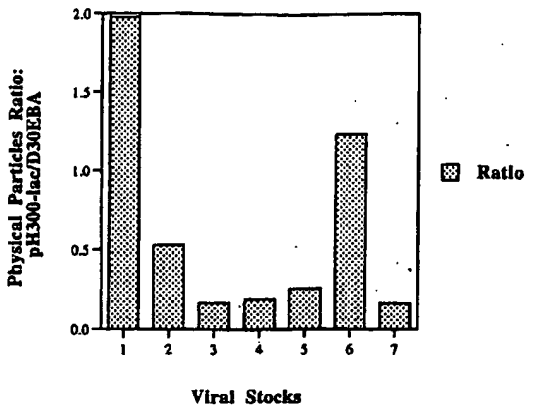

FIG. 2. Slot blot DNA hybridization analysis of the ratio of pH300-lac and helper virus (D30EBA) particles in viral stocks. The pH300-lac-specific probe was a 1.07-kb *Bgl*II-*Eco*RI Hyg^r fragment. The HSV helper-specific probe was the 0.95-kb IE110 promoter *Hin*dIII-*Bam*HI fragment. The radioactive signals were exposed and analyzed on a PhosphorImager (Molecular Dynamics). The ratios were corrected for copy numbers of the probes Hyg^r and IE110 promoter in the packaged multimeric pH300-lac and monomeric HSV-1, respectively.

respectively, were also infected efficiently (Fig. 3C and D). In contrast, maximum transfection efficiencies of 10 to 20% were obtained by lipofection in these cell lines (data not shown). The highest efficiency was obtained with T98G glioblastoma cells (Fig. 3E and F). Figure 4 illustrates the infectivity of pH300-lac on tumor cells derived from different tissues. Tumor cells from breast (T47D, SKBR-3, MCF-7, and DT-20), lung (SW1271), and liver (HepG2) carcinomas were efficiently infected at an MOI of 3 (Fig. 4), while cells from muscle (RD) and kidney (A498) tumors were less efficiently infected (not shown). The results are summarized in Table 2. Most of the cells were efficiently infected in vitro by pH300-lac at an MOI of 3. Therefore, the pH300 miniviral vector can deliver and express transgenes such as *lacZ* with high efficiency in various human cell types.

To demonstrate de novo expression from the mini-EBV/HSV-1 virus, pH300-lac virions were irradiated with various doses of UVC (254 nm) and then used to infect T98G cells. X-Gal staining 24 h after infection showed that the number of X-Gal-positive cells was decreased in response to the UVC doses (data not shown). ONPG measurements taken 1 week after infection showed decreasing *lacZ* expression with increasing UVC doses (Fig. 5). The dose of UVC irradiation needed to reduce *lacZ* expression to 37% (1 lethal hit) was approximately 160 J/M^2. About 1 to 2% X-Gal staining in T98G cells was observed at 4 weeks following infection at an MOI of 3. ONPG measurements of pH300-lac-transduced T98G cells at this MOI showed a peak of *lacZ* gene expression 3 days postinfection, with a continuous decline of ONPG levels thereafter. ONPG levels above that of uninfected controls were obtained up to 5 weeks following infection (data not shown). In these experiments, the transduced cells were cultured in the absence of selection and split at a ratio of 1:4 when confluent. The packaged pH300-lacZ expressed the *lacZ* gene after infection of target cells. Trypan blue staining did not reveal any apparent cytotoxicity in infected cells, even at an MOI of 3. Hence, such infected cells could be easily grown in large quantities for further analysis.

Functional gene delivery in vivo by pH300-lacZ virus into human tumor cells. Infection and expression of pH300-lac in vivo were evaluated in animals (female nude mice from Harlan Laboratories) by in situ injection of pH300-lac virions into HepG2 tumors that were preimplanted by subcutaneous injection (Fig. 6). In vivo infection and expression of pH300-lac was demonstrated by X-Gal staining 24 h after injection of the virus into the tumor tissue. Cells prepared from freshly isolated tumor tissue stained blue, indicating that the *lacZ* gene was delivered to and expressed β-galactosidase in these tumor cells. Among the randomly prepared cryosections, 25% fully expressed *lacZ*, with all areas showing blue staining (Fig. 6A and B); 38% exhibited partial expression, with most areas showing blue staining (Fig. 6C and D); 15% exhibited less expression (Fig. 6E and F), and about 22% of the cryosections showed only scattered blue staining. In contrast, in cryosections prepared from mock-injected control tissue on the same animal, blue staining was not detectable (Fig. 6G and H).

DISCUSSION

An infectious hybrid EBV/HSV-based miniviral vector, pH300, was constructed and used to deliver a reporter gene, *lacZ*, into human cells both in vitro and in vivo. pH300 is capable of high efficient gene transfer and expression in a variety of human cells, both in vitro and in vivo. It also carries

TABLE 2. Infection and expression of pH300-lac in human cells

Cell line	Tissue source	Morphology	Pathology	% X-Gal-positive cells[a]
NHF	Skin	Fibroblast	Normal	90–95
VA13	Lung	Epithelial-like	SV40 transformed	≥95
JML	Skin	Fibroblast	Li-Fraumeni syndrome	90–95
XP4BE	Skin	Fibroblast	DNA repair defect	≥95
GM6914A	Skin	Fibroblast	Fanconi's anemia	80–90
T98G	Nerve	Fibroblast	Glioblastoma	≥95
T47D	Breast	Epithelial	Carcinoma	≥95
SKBR-3	Breast	Epithelial	Carcinoma	≥95
MCF-7	Breast	Epithelial	Carcinoma	80–90
DT-20	Breast	Epithelial	Carcinoma	≥95
RD	Embryonal	Spindle-like	Rhabdomyosarcoma	50–55
SW1271	Lung	Epithelial	Carcinoma	80–90
A498	Kidney	Epithelial	Carcinoma	50–60
HepG2	Liver	Epithelial	Carcinoma	≥95

[a] Cells were infected with pH300-lac at an MOI of 3, and X-Gal staining was carried out 24 h later. Fractions of X-Gal-positive cells were evaluated in at least two independent experiments.

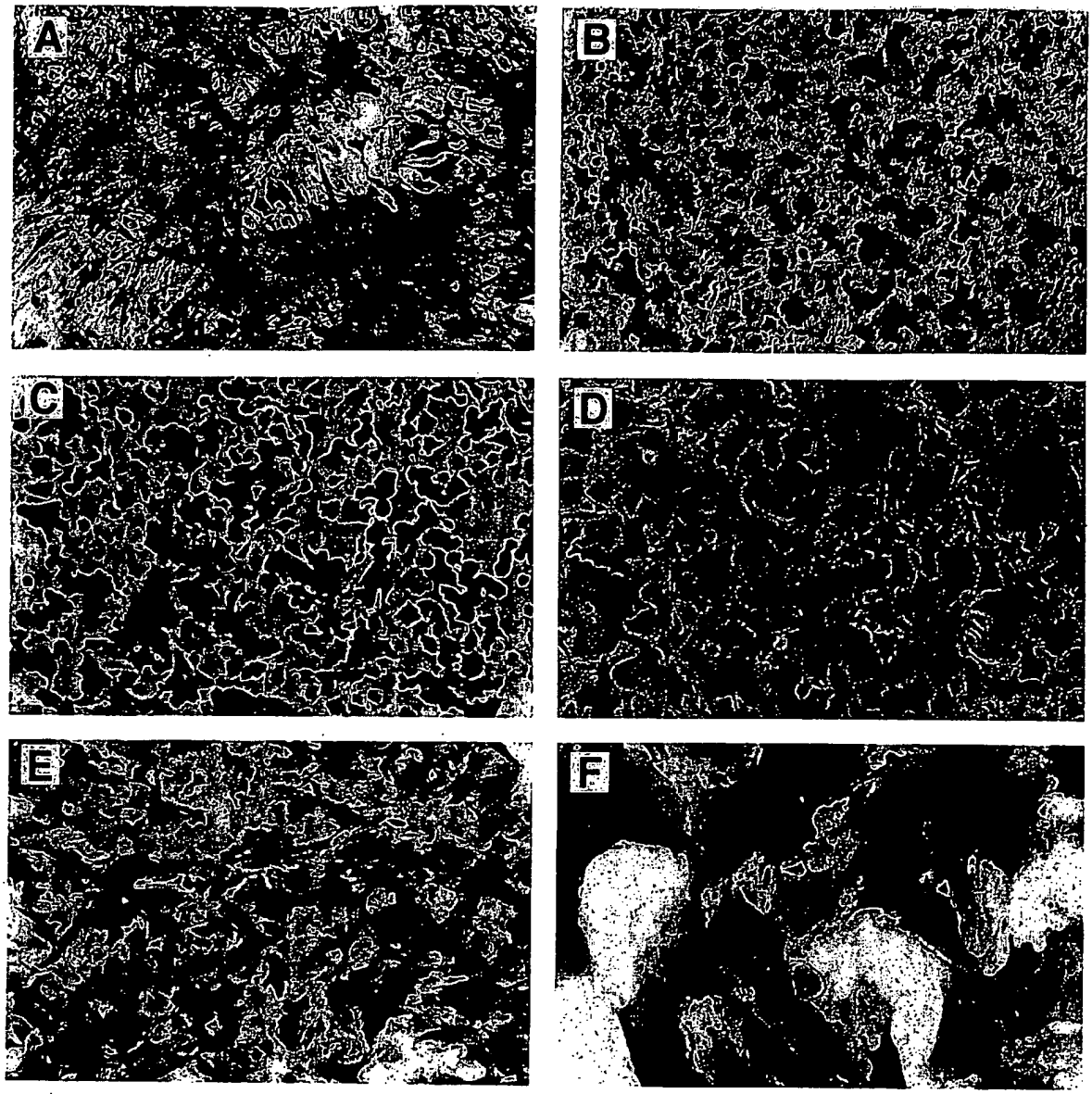

FIG. 3. X-Gal staining of human cells infected in vitro with pH300-lac virions. Cells were infected at an MOI of 3 for 3 h and stained with X-Gal 24 h later. (A) NHF (normal fibroblasts), magnification, ×60; (B) JML (Li-Fraumeni syndrome), magnification, ×60; (C) XP4BE (xeroderma pigmentosum variant), magnification, ×63; (D) GM6914A (Fanconi's anemia), magnification ×95; (E and F) T98G (glioblastoma), magnifications, ×95 and ×304, respectively.

a bacterial plasmid backbone for easy manipulation and amplification in prokaryotic cells.

Latent episomes as a strategy for efficient packaging. One notable characteristic of pH300 is that it contains the latent EBV replication origin, oriP, and its transactivator, EBNA-1. These two elements make the pH300 vector capable of self-replication and mitotic segregation in an episomal form in dividing human cells (22, 29, 35, 36). Episomal maintenance of the vector is an essential step for effective vector replication and packaging into HSV capsids in the presence of HSV-1 helper virus. In addition, the Hyg^r gene ensures that every helper cell contains the miniviral vector and that high titers of viral stock can be made. Under hygromycin selection, helper cells maintained episomal pH300-lac virions could be produced from the helper cells for at least 6 months in culture (unpublished observations). By contrast, the HSV-1 amplicon could not be episomally maintained in helper cells for extended periods of time, and viral stocks had to be produced immediately after transfection (8, 10, 15, 24, 25, 28, 32). Because of low transfection efficiency, only a fraction of cells in the transiently transfected population contained the amplicon. Since the ratio of amplicon to helper virus was very low in the initial passage, higher ratios of viral stocks could be obtained only by successive propagation at a higher MOI for at

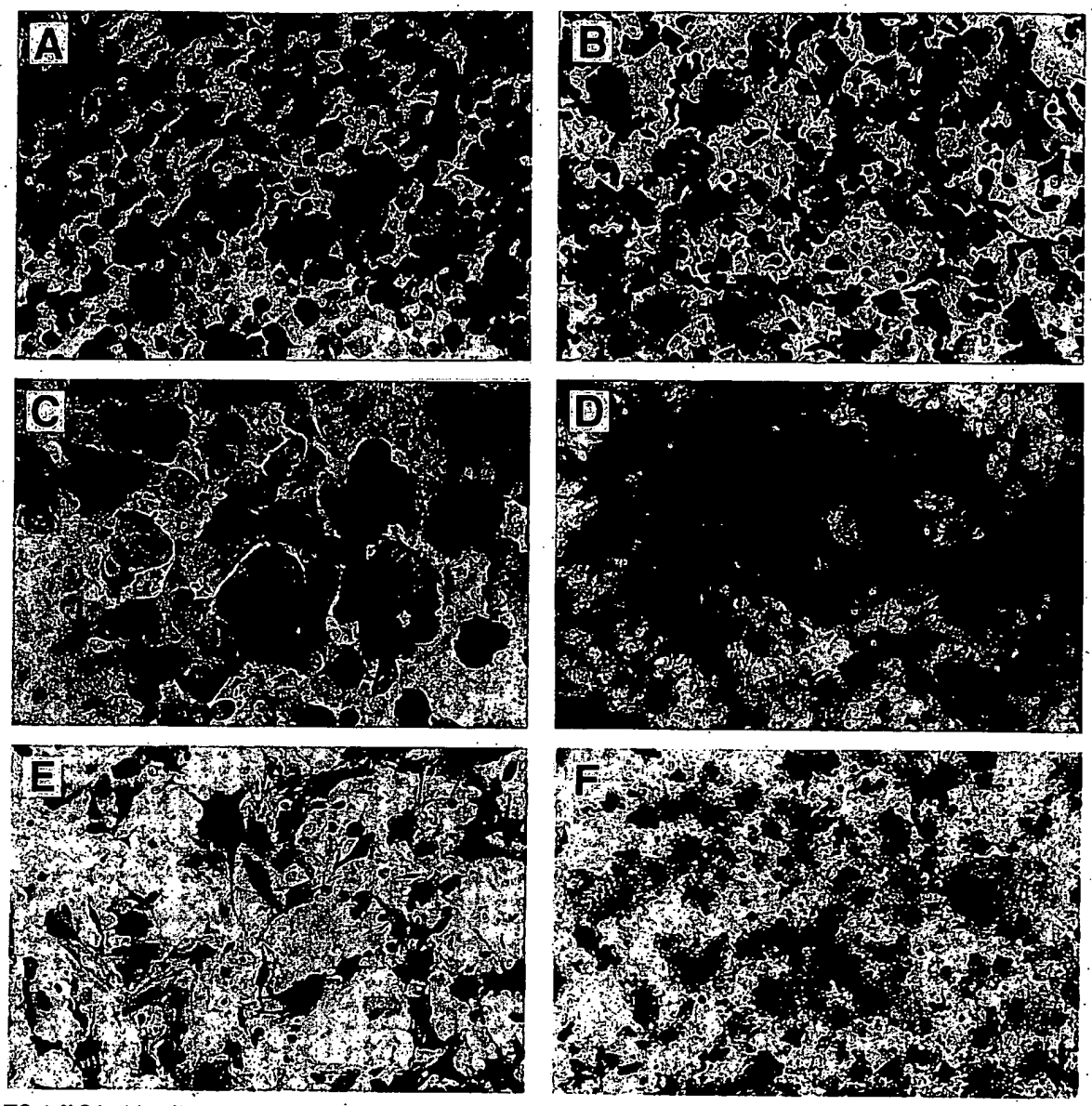

FIG. 4. X-Gal staining of human tumor cell lines infected with mini pH300-lac virions. Cells were infected at an MOI of 3 for 3 h and stained with X-Gal 24 h later. (A) T47D (breast cancer), magnification, ×60; (B) SKBR-3 (breast cancer), magnification, ×60; (C) MCF-7 (breast cancer), magnification, ×190; (D) DT-20 (breast cancer), magnification, ×190; (E) SW1271 (lung cancer), magnification, ×60; (F) HepG2 (liver cancer), magnification, ×60.

least five to eight passages (8, 13, 24). However, what limits the use of this strategy is the apparent difficulty of reproducing such a high ratio of amplicon to helper virus (7). In accordance with these results, the ratio of miniviral pH300-lac vector to helper virus after a single passage of viral stock propagated in helper cells without hygromycin selection was only about 4% (Table 1). In contrast, slot blot hybridization analysis showed that the pH300-lac/D30EBA ratios were in the range of 1.98 to 0.165 (Fig. 2), while biological titration indicated ratios of packaged pH300-lac to D30EBA varying between 15 and 0.256 (Table 1). A lower ratio of pH300-lac to helper by biological titration than by molecular hybridization analysis may be due to the fact that the lysis plaque assay requires multiple rounds of viral cycles in E5 cells, while the *lacZ*-based assay relies on a single infection cycle. Titers of pH300-lac virions, however, are reproducible because expression of the *lacZ* gene is more direct than viral titration based on lysis plaques.

Hybrid mini-EBV/HSV-1 for reduced helper interference. The pH300-lac is defective for viral production for both EBV and HSV-1 and is thus dependent on a helper virus to provide all needed viral proteins in *trans*. The helper HSV-1 (D30EBA) used for pH300-lac production is also replication defective and incapable of lytic infection as a result of a deletion in both copies of the IE 3 gene. Viral functions of IE3 are

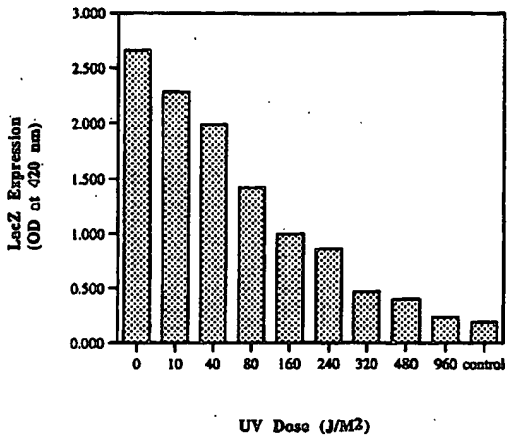

FIG. 5. Effect of UVC on infection and expression of pH300-lac in cultured human glioblastoma T98G cells. The viral stock was irradiated with various doses of UVC as indicated and then used to infect T98G cells. The β-galactosidase activity of miniviral pH300-lac was determined 1 week later by the measurement of optical density (OD) at 420 nm. Each bar represents the mean value of three independent determinations of ONPG.

essential for early gene expression, viral DNA replication, late gene expression, and thus the production of progeny virus (6). Consequently, the helper virus can be replicated and propagated only through the lytic cycle in helper cells such as E5 expressing the IE3 gene through complementation. Human cells do not contain a homologous IE3 gene and are therefore not permissive for HSV helper virus replication. However, the regeneration of wild-type HSV-1 through recombination between the helper viral genome and the chromosomally integrated complementing viral genes during in vitro passage has been reported (11). In contrast, we have not been able to detect any rescued wild-type virus in our experiments (data not shown). Such observation may be due to a combination of the low frequency of such recombination events (10^{-6} to 10^{-7} in reference 11) and the usage of a single short-term infection cycles at low MOI of helper for making viral stocks (instead of multiple repetitive passages at high MOI of helper). As additional safety precautions, several strategies can be envisioned to reduce the probability of recombination in vitro or in vivo, such as the engineering of a cell line expressing multiple viral genes codeleted from a helper virus (10a) or the development of complementing viral subgenomes to passage a fragmented helper virus (7a). Our observations indicate that there were no apparent cytopathic effects after infection of human cells with pH300-lac virions in vitro. In a recent study, Johnson et al. (11) detected cytotoxicity in cells infected with a replication-defective mutant (IE3 deletion) of HSV-1, CgalD3. In confirmation, the helper virus only (D30EBA) caused cell death 3 days after infection (unpublished observations). The miniviral system pH300 also carries helper virus in viral stocks. Specifically, the pH300-lac viral stocks consisted of an average vector/helper ratio of 0.74 (Table 1, viral stocks 2 to 7), with the noticeable exception of a ratio of 15.0 for viral stock 1. In contrast, a ratio of 0.04 was observed when the traditional method of transient transfection of the amplicon was used (viral stock 8). Hence, the presence of helper in the pH300 system was reduced by ca. 18-fold (0.74/0.04). Such a lower amount of helper virus should help reduce the cytopathic effects observed after infection with HSV-1 recombinants or previous HSV amplicons. In addition, the lower amount of helper virus could also diminish potential immune reactions to viral components in vivo. However, the usage of helper HSV to generate pH300 viral stocks still presents the disadvantage of some helper contamination, which could preserve some virus-induced *in vivo* cytotoxicity. Such a potential cytotoxicity of HSV is a major concern, which could be reduced if not completely eliminated through the development of helper virus-free packaging systems for HSV vectors.

Multimeric packaging for efficient gene expression. The EBV/HSV-1 hybrid vector pH300 is capable of infecting a variety of types of human cells, both in vitro and in vivo (Fig. 3 to 5). The β-galactosidase activity detected after infection of pH300-lac may not necessarily derive from de novo expression of the transduced *lacZ* gene, since the β-galactosidase protein may be encapsidated into helper and/or defective virions during the packaging process. To exclude such a possibility, we examined the effect of UVC irradiation on β-galactosidase activity after infection. As shown in Fig. 6, β-galactosidase activity of miniviral pH300-lac was inhibited by UV irradiation in proportion to the dose (Fig. 6), demonstrating that β-galactosidase activity of the *lacZ* gene was indeed conferred by the transduced pH300-lac DNA and due to de novo expression. Because the pH300-lac DNA is packaged into HSV particles, its tropism of infection is probably as wide as that of the wild-type HSV-1. We have also successfully infected primary mouse liver cells with pH300-lac and achieved about 95% X-Gal-stained cells (34a). Such a result indicates a host range wider than human cells. However, episomal replication of the vector is not expected to occur in rodent cells since the oriP/EBNA-1 replication system from EBV is limited essentially to human and primate cells. For certain types of human cells such as RD and A498, derived from muscle and kidney, respectively, infection efficiency was somewhat reduced. This could be due to a lower number of HSV-1 receptors on the membranes of those cells. Alternatively, such cell types may not sustain efficient expression from the CMV immediate-early promoter. Our data indicated that *lacZ* gene expression generally lasted for approximately 2 weeks in the majority of human cell lines tested. In T98G cells, β-galactosidase activity could be detected for up to 5 weeks after in vitro infection (data not shown). Such a decrease of β-galactosidase activity as a function of time in infected cells might also be due to promoter inactivation or, alternatively, to vector loss in the actively growing cells. The very intense staining of cells infected both in vitro and in vivo indicates that the CMV promoter was very active. Because of the smaller size of the pH300-lac and its lytic replication via a rolling circle, a mini-HSV-1 vector is packaged as linear multimeric concatemers consisting of identical head-to-tail repeat units (24). Hence, the number of multimers in a virion is dictated by the size ratio of the monomeric vector to the overall 152 kb of HSV-1 DNA (33). Such a multimeric structure may also contribute to high expression levels per cell. By using pulsed-field gel electrophoresis and Southern blot methods, Sun and Vos (31) measured a packaging size range of 150 to 200 kb for a multimeric mini-EBV, another member of the human herpesvirus family. Hence, the pH300-lac vector is expected to be packaged as 8-mers into HSV-1 viral particles. The maximum size of insert DNA that can be packaged into mini-HSV-1 viral capsids is not known. Theoretically, insertion of nonviral sequences into mini-HSV-1 as a monomeric vector may reach the total size of the viral genome. In the case of pH300, the theoretical maximum insertion size of foreign DNA might be as large as 130 to 140 kb.

Effective in vivo delivery in tumors as a potential tool for gene therapy. Successful infection of tumor cells in vivo (Fig. 5) by in situ injection of pH300-lac virions indicates that the miniviral pH300 has potential use for human tumor treatment

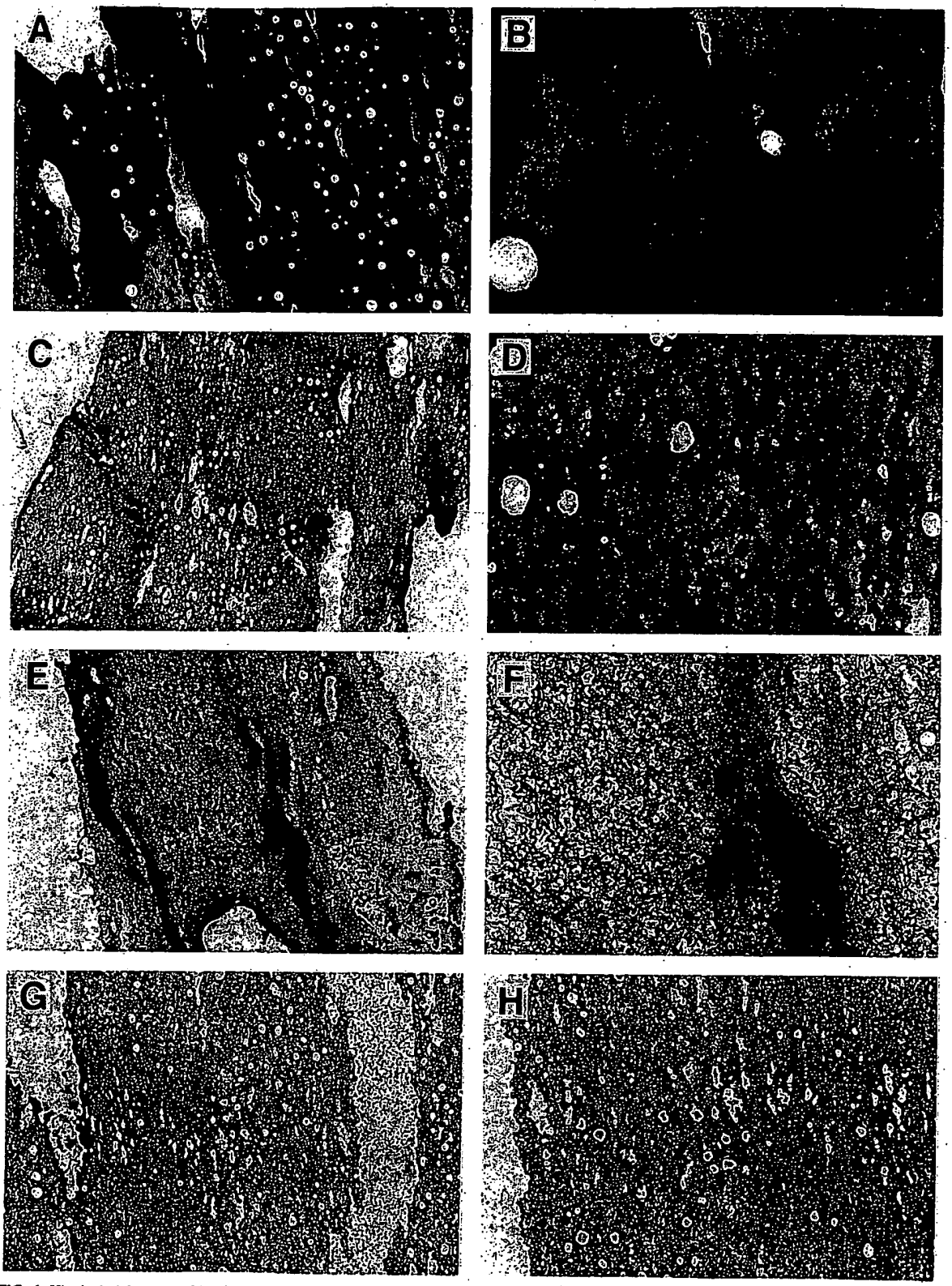

FIG. 6. Histological detection of β-galactosidase activity in nude mouse tumors 24 h after pH300-lac injection into human tumor cells grown in nude mice. (A and B) Area with high *lacZ* expression; (C and D) area with medium *lacZ* expression; (E and F) area with less *lacZ* expression; (G and H) control area. Magnifications: A, C, E, and G, ×19; B, D, F, and H, ×190.

in vivo. The observed strong and transient expression of an inserted gene in pH300 may be sufficient in killing tumor cells. Alternatively, the mini-HSV-1 vector could be used in the central nervous system for treating acquired and hereditary diseases due to the neurotropism of HSV-1. Geller and Breakefield (8) explored the feasibility of the mini-HSV-1 amplicon to transfer expressed genes into nervous cells. A follow-up study by Kaplitt et al. (13) also using the *lacZ* gene on an HSV-1 amplicon confirmed mini-HSV-1 as a promising viral vector for gene transfer and expression in the central nervous system of the adult rat brain in vivo. Preexisting or future HSV infection could theoretically induce vector reactivation and its spread to other parts of the human body. This issue is a primary concern for future gene therapy protocols based on any infectious vector derived from endemic human viruses. Such potential pathogenesis induced by rescued infectious HSV vector spreading at new sites would not be expected to be as severe as that of wild-type virus infection, since all herpesvirus genes have been deleted from the vector. Nonetheless, strategies such as controlled tissue-specific gene expression and inclusion of suicide genes for viral destruction will have to be considered as safety barriers. In summary, the wide tropism of infection, the simplicity of handling, the potentially large capacity of DNA insertion, the undetectable cytotoxicity in vitro, and the high efficiency of infection and expression in vivo may render the EBV/HSV-1 hybrid vector pH300 a potentially excellent gene transfer system for future gene therapy applications.

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Vaccinia topoisomerase and Cre recombinase catalyze direct ligation of activated DNA substrates containing a 3′-*para*-nitrophenyl phosphate ester

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ABSTRACT

DNA topoisomerases and DNA site-specific recombinases are involved in a diverse set of cellular processes but both function by making transient breaks in DNA. Type IB topoisomerases and tyrosine recombinases cleave DNA by transesterification of an active site tyrosine to generate a DNA–3′-phosphotyrosyl–enzyme adduct and a free 5′-hydroxyl (5′-OH). Strand ligation results when the 5′-OH attacks the covalent complex and displaces the enzyme. We describe the synthesis of 3′-phospho-(*para*-nitrophenyl) oligonucleotides (3′-pNP DNAs), which mimic the natural 3′-phosphotyrosyl intermediate, and demonstrate that such pre-activated strands are substrates for DNA ligation by vaccinia topoisomerase and Cre recombinase. Ligation occurs by direct attack of a 5′-OH strand on the 3′-pNP DNA (i.e., without a covalent protein–DNA intermediate) and generates free *para*-nitrophenol as a product. The chromogenic DNA substrate allows ligation to be studied in real-time and in the absence of competing cleavage reactions and can be exploited for high-throughput screening of topoisomerase/recombinase inhibitors.

INTRODUCTION

DNA topoisomerases and site-specific DNA recombinases carry out cleavage and ligation reactions involving DNA phosphodiester bonds. Type IB topoisomerases and members of the λ integrase family of recombinases (also called tyrosine recombinases) have similar tertiary structures and reaction mechanisms (1–6). Strand cleavage by these enzymes is the result of transesterification of an active site tyrosine nucleophile to one strand of duplex DNA to generate a covalent DNA–(3′-phosphotyrosyl)–enzyme intermediate and a 5′-hydroxyl (5′-OH) DNA leaving group. Strand ligation is the result of a second transesterification event in which a 5′-OH attacks the covalent intermediate and displaces the enzyme (Fig. 1A). The 3′-covalent adduct is transient because the rate of cleavage (k_{cl}) exceeds the rate of religation (k_{rel}). In a typical topoisomerase reaction, the covalently bound monomeric protein releases the 5′-OH strand and permits it to swivel about the opposing phosphodiester on the complementary strand before catalyzing religation, causing a change in DNA linking number. In the case of recombinases, the reactive 5′-OH for religation does not come from the original cleaved DNA strand but from a partner strand cleaved by a second recombinase protomer within a synaptic complex. Paired strand exchanges by recombinases result in the formation and resolution of Holliday junctions.

These two pathways are not mutually exclusive; many tyrosine recombinases have topoisomerase activity (7–9) and type IB topoisomerases can catalyze intermolecular strand ligation and Holliday junction resolution (10–12). In addition, recent studies have shown that the catalytic repertoire of these enzymes is not limited to DNA strand exchange, but embraces transesterification and ligation reactions involving RNA as well as non-nucleic acid nucleophiles (13–20). These 'unconventional' reactions highlight a key theme in the evolution of phosphoryl transfer enzymes, i.e., that relatively subtle changes of the enzyme active site or the structure of the nucleic acid substrate can convert a topoisomerase/recombinase into a DNA endonuclease, an RNA endonuclease or a polynucleotide ligase.

Site-specific DNA recombinases are involved in a large number of cellular functions including integration/excision of mobile DNA elements, control of gene expression, resolution of dimeric DNA chromosomes, and the generation of genetic diversity. Topoisomerases play a major role in maintaining proper DNA topology during transcription and replication, and are targets for anti-tumor drugs such as camptothecin. Mechanistic studies of these important enzymes depend on the ability to accurately measure the forward and reverse transesterification reactions in isolation. For example, suicide cleavage substrates have been of enormous value in studying the structural basis for transesterification chemistry. One class of suicide substrates incorporates a 5′-bridging phosphorothiolate linkage at the scissile phosphate (6,21–24). This modification generates a 5′-sulfyhydryl (5′-SH) leaving strand instead of a 5′-OH following transesterification of the active site tyrosine. Because the 5′-SH is an incompetent nucleophile for the religation reactions, the enzyme–DNA covalent adduct is trapped at the nick.

Substrates that allow exclusive focus on the religation reaction are not as readily available. In some topoisomerase

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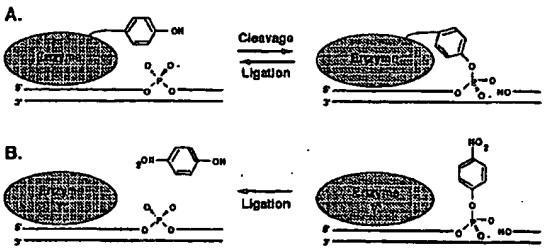

Figure 1. Elementary topoisomerase reactions and rationale for suicide substrates. (A) DNA strand cleavage results from attack of an active-site tyrosine residue forming a covalent 3′-phosphotyrosine–DNA intermediate. DNA strand ligation results from attack of the displaced 5′-OH and expulsion of the active site tyrosine. (B) Pre-activated 3′-pNP DNA is illustrated on the right. Enzyme-mediated transesterification of the 5′-OH will expel *para*-nitrophenol. If a mutant enzyme lacking the tyrosine nucleophile is used, then competing cleavage reactions cannot occur.

systems it has been possible to measure the rate of strand ligation independent of strand cleavage by assaying the ability of an exogenous oligonucleotide to attack a pre-activated enzyme–DNA covalent complex under single-turnover conditions (25). This analysis is predicated on the ability to form the covalent intermediate in high yield and, ideally, to isolate the enzyme–DNA adduct. These conditions are not readily applicable to recombinases because strand ligation occurs in a synaptic complex of four recombinase protomers and four DNA cleavage sites. In addition, very few systems are sufficiently well-defined kinetically to ensure that the reactions occur under single-turnover conditions.

An alternative approach is to chemically synthesize pre-activated DNA ligation substrates. For example, Sadowsky and colleagues (26,27) demonstrated that Flp recombinase can join DNA strands at a nick without formation of a covalent enzyme–DNA intermediate, provided that the 3′-phosphate (3′-PO_4) terminus at the nick is chemically activated by esterification to a tyrosine residue. Mutation of the Flp active site Tyr343 to Phe had no apparent effect on the high efficiency with which such activated ends were sealed. The latter result demonstrated that ligation can be assayed in the absence of competing cleavage reactions, because the free tyrosine product diffuses away from the active site and the Flp Y343F mutant cannot recleave the ligated strand. Jayaram and colleagues (28,29) have since shown that Flp normally acts through a *trans* cleavage mechanism, whereby one molecule of Flp binds the DNA target site and catalyzes attack by the tyrosine of a second Flp monomer on the scissile phosphate. Thus, the activation of the 3′-end via a phosphodiester to a single tyrosine is essentially equivalent to the natural covalent intermediate formed during FLP-mediated strand transfer.

Cre recombinase and mammalian topoisomerase I are also capable of sealing DNA nicks with 3′-phosphotyrosine/5′-OH termini, albeit much less efficiently than Flp (26,27). Given the crystallographic evidence that Cre and mammalian topoisomerase I normally cleave DNA *in cis* (1,6), their low efficiency of ligation of a 3′-phosphotyrosine-activated strand may reflect steric clashes of the extra tyrosine at their respective active sites. The capacity of active site mutants of Cre and mammalian topoisomerase I to ligate 3′-phosphotyrosine-activated strands was not examined. It is therefore possible that replacement of tyrosine with a much smaller side chain might free up space to accommodate a DNA-bound tyrosine. Esterification of DNA to lower molecular weight tyrosine analogs might also circumvent steric constraints at the active site.

We have developed a post-synthetic method for attaching a relatively small tyrosine analog, *para*-nitrophenol, onto the 3′-end of oligonucleotides. We demonstrate here that the resulting pre-activated 3′-phospho-(*para*-nitrophenyl) oligonucleotides (3′-pNP) support DNA strand ligation by wild-type vaccinia topoisomerase (a prototypal type IB enzyme) and Cre recombinase and as well as by mutated versions containing Phe or Ala in lieu of the nucleophilic tyrosine on the enzyme. Hence, DNA ligation occurs in the absence of any competing DNA cleavage reactions (Fig. 1B). The 3′-pNP substrates are also useful because the ligation reaction releases *para*-nitrophenol, which can be assayed spectrophotometrically. This important feature allows DNA strand ligation to be assayed in real time and has implications for developing high-throughput assays for elementary topoisomerase reactions.

MATERIALS AND METHODS

Synthesis of 3′-pNP DNA

Oligonucleotides (1 μmol scale) were synthesized using 3′-PO_4 CPG (Glen Research, Sterling, VA) and standard DNA phosphoramidites on an ABI 392 automated DNA synthesizer. After the last coupling step, the final 5′-dimethoxytrityl protecting group (5′-DMT) was not removed so that the final oligonucleotide contained a 3′-PO_4 but lacked a free 5′-OH. The resin was incubated in concentrated ammonia at 55°C for 12 h and the resulting supernatant was dried *in vacuo* to a powder. The powder was dissolved in 250 μl of 2 mM $MgCl_2$, 100 mM MES (pH 5.5) and any insoluble material was removed by centrifugation. Aliquots of 200 μl of 3 M *para*-nitrophenol (Aldrich, Milwaukee, WI) in acetonitrile and 0.048 g of 1-[3-(dimethylamino)propyl]-3-ethycarbodiimide hydrochloride (EDC) (Aldrich) were added sequentially. The resulting two immiscible layers where shaken vigorously in the dark at room temperature to form an emulsion. After 12–16 h, 250 μl of water was added and the aqueous layer was extracted three times with 500 μl each of ethyl acetate. The oligonucleotide was precipitated with the addition of 0.1 vol of 3 M sodium acetate and 3 vol of absolute ethanol. The resulting pellet was resuspended in 250 μl of 80% glacial acetic acid at 0°C and immediately precipitated with the sequential addition of 50 μl of 3 M sodium acetate and 900 μl of absolute ethanol.

The 3′-derivatized DNA (3′-pNP DNA) was purified by reverse phase chromatography on a Hewlett Packard 1050 HPLC using a RP-318 250 mm × 4.6 mm column (Bio-Rad, Hercules, CA) at 1 ml/min, 5→25% acetonitrile gradient (buffered with 100 mM triethylammonium acetate pH 7.0) over 30 min. Peak fractions (0.5 ml) were pooled, concentrated *in vacuo*, and stored in 1 mM Tris (pH 7.5), 0.1 mM EDTA at –20°C.

Purification of vaccinia topoisomerase

Wild-type (WT) vaccinia topoisomerase and mutated versions Y274F, Y274A and R223A were expressed in *Escherichia coli* BL21 cells by infection with bacteriophage λCE6 (30) and

then purified from a soluble bacterial lysate by phosphocellulose column chromatography. The protein concentrations of the phosphocellulose preparations were determined by using the Bio-Rad dye-binding reagent with bovine serum albumin as the standard.

Topoisomerase substrate preparation

DNA oligonucleotides containing the 3′-pNP were 5′-end-labeled with [γ-^{32}P]ATP and purified by electrophoresis through a 20% polyacrylamide gel. The labeled oligonucleotides were eluted from an excised gel slice and then hybridized to unlabeled downstream oligonucleotides and complementary oligonucleotides at a ratio of 1:4:4. Annealing reaction mixtures containing 0.2 M NaCl and oligonucleotides were heated to 70°C and then slow-cooled to 22°C. For spectrophotometric measurement of the release of *para*-nitrophenol, the 3′-pNP oligonucleotide was annealed to the downstream oligonucleotide and complementary oligonucleotide at a molar ratio of 1:2:2 in the absence of NaCl. The hybridized DNAs were stored at 4°C.

Purification of Cre recombinase

WT Cre recombinase and mutated versions Y324F, Y324A and Y324V were expressed in *E.coli* BL21(DE3) cells with the addition of 1 mM IPTG and then purified from a soluble bacterial lysate by phosphocellulose column chromatography as previously described (31).

Recombinase substrate preparation

DNA oligonucleotides containing the 3′-pNP moiety were 5′-end-labeled with [γ-^{32}P]ATP and purified by electrophoresis through a 20% polyacrylamide gel or by passage through a Bio-gel P-10 (Bio-Rad) spin column. The labeled oligonucleotides were hybridized to unlabeled downstream oligonucleotides and complementary oligonucleotides at a ratio of 1:2:10. Annealing reaction mixtures containing 0.1 M KCl and oligonucleotides were heated to 90°C and then slow-cooled to room temperature.

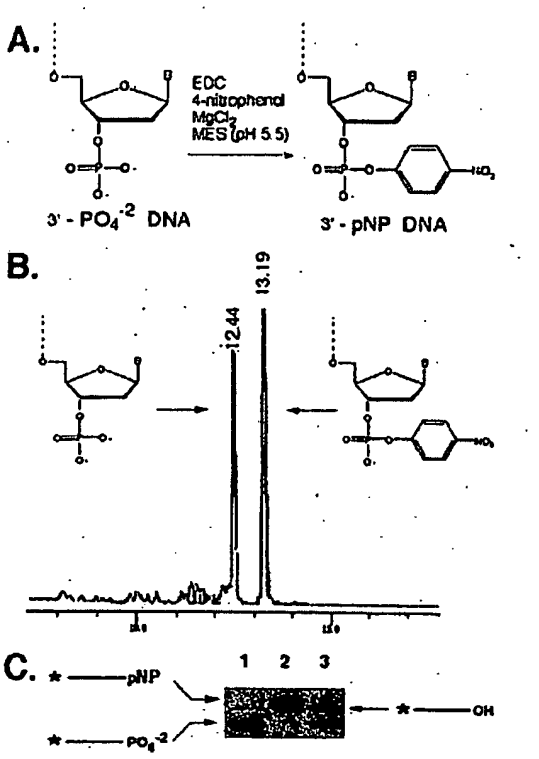

Figure 2. Synthesis of 3′-pNP DNA. (A) The 3′-terminal phosphate of an oligonucleotide is illustrated on the left; condensation with *para*-nitrophenol yields 3′-pNP DNA, illustrated on the right. (B) 3′-PO_4 and 3′-pNP DNA (23mer) were resolved by reverse phase chromatography (see Materials and Methods). The resulting chromatogram (A_{260} versus time) is shown. (C) 5′-end-labeled 23mer containing a 3′-PO_4^{2-} (lane 1), 3′-pNP (lane 2), or 3′-PO_4^{2-} and 3′-OH mixture (lane 3) were resolved on a 20% acrylamide, 8 M urea, 0.5× TBE (45 mM Tris–borate, 1.25 mM EDTA) 0.4 mm sequencing gel. The resulting autoradiogram is shown.

RESULTS

Synthesis of 3′-pNP oligonucleotides

Previously described methods for synthesis of 3′-phosphotyrosyl DNA oligonucleotides relied upon serial 3′ to 5′ addition of standard base-protected nucleotide building blocks to a tyrosine-derivatized resin (32). This approach is limited because other tyrosine analogs cannot be easily attached to the resin and incorporation of different tyrosine analogs would require separate chemical synthesis of different resins. We have investigated the alternative approach of derivatizing oligonucleotides post-synthetically to produce 3′-modified DNA. The advantage of this approach is that a single oligonucleotide can be derivatized with different reagents so that a more diverse set of analogs can be attached to the 3′-end of the DNA.

para-Nitrophenol was chosen for several reasons. First, this analog is significantly smaller than tyrosine (139 versus 181 g/mol). As discussed above, the pre-activated substrate must be accommodated within the enzyme active site and *para*-nitrophenyl derivatized DNA (3′-pNP) might be expected to cause less steric clash. Second, the electron withdrawing *para*-nitro moiety makes this analog a better leaving group than tyrosine. Finally, free *para*-nitrophenol, but not 3′-pNP DNA, absorbs 400–405 nm wavelength light. This allows the ligation reaction to be monitored in real time because the ligation reaction is predicted to generate free *para*-nitrophenol (Fig. 1B). The 3′-pNP DNA substrate is therefore similar to *para*-nitrophenyl-thymidine 3′-phosphate, a substrate used to assay spleen phosphodiesterase and enzymes of similar specificity (33).

The post-synthetic approach for modifying the 3′-end of oligonucleotides detailed in Figure 2A was a modification of a protocol used to derivatize nucleotides and oligonucleotides (34). The water-soluble condensing agent, EDC, was used to specifically modify the 3′-PO_4 of DNA (Fig. 2A). Using optimized conditions, ~60% of the 3′-PO_4 DNA was converted to 3′-pNP DNA after 12 h. No product was formed when a 3′-OH terminated oligonucleotide was used (data not shown). No detectable side products accumulated after 12 h, however side products were detected by reverse phase HPLC analysis (see below) after 24 h. One potential side reaction is the condensation

of a 5′-OH and the 3′-PO_4. In order to minimize this reaction, the 5′-DMT group was not cleaved from the oligonucleotide following the last coupling reaction. The 5′-DMT group was removed following the EDC condensation reaction and before subsequent purification steps. The 5′-DMT may also improve the solubility of the oligonucleotide in the condensation reaction.

The 3′-pNP DNA is more hydrophobic than the starting material and can be purified by reverse phase HPLC. To demonstrate this, equal amounts of 3′-PO_4 and purified 3′-pNP DNA were mixed and then separated by reverse phase HPLC. A portion of the resulting chromatogram is shown in Figure 2B. When a sample of the later-eluting material (3′-pNP DNA) was incubated with spleen phosphodiesterase, the reaction product strongly absorbed 400 nm light. Incubation of the sample with venom phosphodiesterase did not generate material that absorbed 400 nm light. These results indicate that *para*-nitrophenol was attached to the 3′-PO_4 (data not shown). We have successfully derivatized and purified oligonucleotides of different sequence composition and lengths (from 6- to 45mer). We estimate that 3′-PO_4 and 3′-pNP oligonucleotides up to 50 nt in length could be separated using these methods.

Addition of *para*-nitrophenol to the DNA 3′-terminus would also be expected to increase the molecular weight of the resulting oligonucleotide. To test this prediction, 3′-PO_4 DNA and 3′-pNP DNA (25mer) were 5′-end-labeled and analyzed by electrophoresis through a 20% polyacrylamide gel. An autoradiogram of the gel is shown in Figure 2C. As expected, the 5′-labeled 3′-pNP DNA (lane 2) migrated more slowly through the gel than 5′-labeled 3′-PO_4 DNA (lane 1). In lane 3, a mixture of 5′-labeled 3′-PO_4 and 3′-OH DNAs was resolved. The 3′-OH DNA migrated slower than the 3′-PO_4 DNA and only slightly faster than the 3′-pNP DNA. This result demonstrates that the slower mobility of 3′-pNP versus 3′-PO_4 DNA resulted from an increase in molecular weight and a decrease in charge (3′-PO_4 versus 3′-phosphodiester). This pattern is very similar to polyacrylamide gel electrophoresis (PAGE) analysis of 3′-phosphotyrosine derivatized oligonucleotides (35) and is expected from the relatively small size of the *para*-nitrophenyl group.

Vaccinia topoisomerase catalyzes ligation of a 3′-pNP/5′-OH nick

Vaccinia topoisomerase is a prototype of the type IB topoisomerase family (36). The poxvirus topoisomerase is distinguished from the eukaryotic nuclear topoisomerase I by its compact size (314 amino acids) and its site-specificity in DNA transesterification. Vaccinia topoisomerase binds and cleaves duplex DNA at a pentapyrimidine target sequence 5′-(T/C)CCTT↓ (37). The T↓ nucleotide is linked to Tyr274 of the enzyme. The individual rate constants for cleavage and religation of DNA containing a single CCCTT target site have been measured under single-turnover and equilibrium conditions (25,38–40). Indeed, vaccinia topoisomerase is the only member of the type IB topoisomerase/tyrosine recombinase superfamily for which a detailed kinetic scheme is available.

Can vaccinia topoisomerase catalyze attack of a properly positioned 5′-OH strand on a CCCTTp target site that has been chemically pre-activated by esterification to *para*-nitrophenol (3′-pNP)? To test if this reaction was possible, a 5′-^{32}P-labeled 18mer 3′-pNP oligonucleotide pCATATCCGTGTCGCCCTTpNP

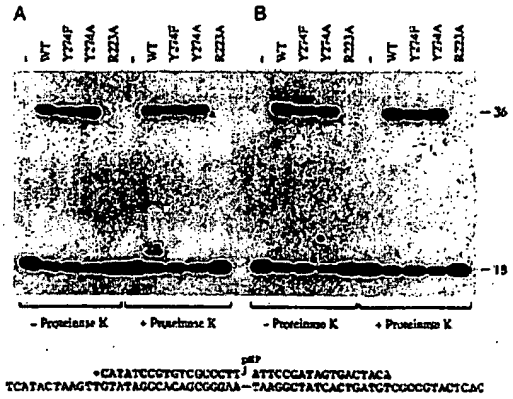

Figure 3. Ligation activity of vaccinia topoisomerase does not require the active site nucleophile Tyr274, but requires catalytic residue Arg223. Reaction mixtures containing (per 20 µl) 50 mM Tris–HCl (pH 7.5), 0.5 pmol of the nicked 3′-pNP/5′-OH DNA substrate (illustrated at the bottom of the figure), and 2.5 pmol of topoisomerase (WT, Y274F, Y274A or R223A) were incubated at 37°C for 24 h. Topoisomerase was omitted from control reaction mixtures (–). Duplicate aliquots (20 µl) were then withdrawn and either quenched immediately with SDS (A) or adjusted to 0.5 M NaCl and incubated at 22°C for 30 s prior to quenching with SDS (B). The samples were then digested with proteinase K where indicated. The DNA was ethanol-precipitated and then analyzed by electrophoresis through a 15% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris–borate, 2.5 mM EDTA). An autoradiogram of the gel is shown. The positions of the 5′-^{32}P-labeled 18mer 3′-pNP strand and the 36mer ligation product are indicated. Note that labeled DNA covalently linked to the full-length topoisomerase polypeptide did not enter the gel and was therefore not seen in the autoradiogram.

was prepared and hybridized to a complementary 60mer strand and a 5′-OH 18mer acceptor strand to form the nicked duplex substrate shown in Figure 3. Reaction of this molecule with a 5-fold molar excess of WT topoisomerase for 24 h at 37°C resulted in the formation of a novel radiolabeled 36mer ligation product that was well-resolved by PAGE from the input ^{32}P-labeled 18mer strand (Fig. 3A). Digestion of the reaction products with proteinase K prior to PAGE resulted in the appearance of a doublet of labeled species migrating at ~20 nt. This cluster corresponded to the 5′-^{32}P-labeled 18mer strand covalently linked to Tyr274 plus one or more flanking amino acids (18). Note that the covalent adduct of full-length topoisomerase bound to 18mer DNA did not migrate into the gel and was therefore not visualized in Figure 3. We did not detect the covalent DNA–peptide adducts when the reaction mixture was adjusted to 0.5 M NaCl after the reaction with topoisomerase and immediately prior to proteinase K digestion (Fig. 3B). It is well documented that high ionic strength will elicit strand closure by topoisomerase bound to an equilibrium CCCTT substrate, by virtue of instantaneous salt-mediated dissociation of the topoisomerase from DNA as soon as it catalyzes strand religation (40,41). The apparent decay of the covalent adduct upon transient exposure to NaCl after prolonged reaction with the 3′-pNP/5′-OH substrate underscores that the enzyme remained catalytically active. This experiment showed that vaccinia topoisomerase can ligate a nick containing a 3′-pNP phosphodiester.

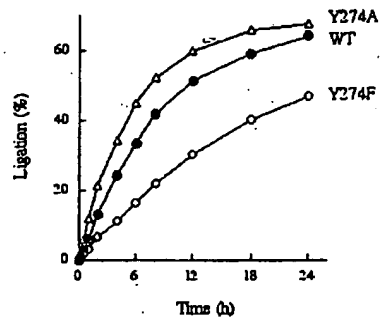

Figure 4. Kinetics of ligation by vaccinia topoisomerase. Reaction mixtures containing (per 20 µl) 50 mM Tris–HCl (pH 7.5), 0.5 pmol of nicked 3′-pNP/5′-OH DNA substrate and 2.5 pmol of topoisomerase (WT, Y274F or Y274A) were incubated at 37°C. The reactions were initiated by addition of topoisomerase. Aliquots were withdrawn at the times specified and incubated with NaCl to 0.5 M final concentration for 30 s. The reactions were then quenched by adding SDS to 0.5%. The samples analyzed by electrophoresis through a 15% polyacrylamide gel containing 7 M urea in TBE. The extent of ligation [36mer/(18mer + 36mer)] was quantitated by scanning the gel using a FUJIX Bio-Imaging Analyzer and plotted as a function of time.

Ligation of a 3′-pNP/5′-OH nick does not require the active site tyrosine

The vaccinia topoisomerase mutants Y274F and Y274A bind to the CCCTT target site, but absolutely cannot transesterify to the scissile phosphodiester to form a covalent intermediate. Therefore, it was striking that the Y274F and Y274A proteins did catalyze the ligation of the nicked 3′-pNP/5′-OH substrate to form a ^{32}P-labeled 36mer product. The extent of strand joining during a 24 h reaction was grossly comparable for WT, Y274F and Y274A proteins (Fig. 3A). The DNA–peptide adduct formed after proteolytic digestion of the WT topoisomerase reaction products was not detected when the Y274F and Y274A ligation products were treated with proteinase K (Fig. 3A), consistent with the identification of the ~20mer cluster as a degradation product of the covalent topoisomerase–DNA complex.

At 125 nM enzyme and 25 nmol DNA, the Y274A ligation reaction displayed pseudo first-order kinetics (Fig. 4). An endpoint of 70% of the input labeled 3′-pNP strand converted to 36mer was attained at 18–24 h. The data fit well to a single exponential over three half-lives with an apparent rate constant for 3′-pNP/5′-OH ligation of 5×10^{-5} s^{-1}. This value is at least four orders of magnitude slower than the rate constant for topoisomerase-catalyzed attack of 5′-OH DNA on the covalent DNA–(3′-phosphotyrosyl)–enzyme intermediate ($k_{rel} \sim 1$ s^{-1}). The ligation reaction of WT topoisomerase attained a similar endpoint and the apparent rate constant (3.3×10^{-5} s^{-1}) was about half that of the Y274A mutant (Fig. 4). Thus, elimination of the bulky tyrosine side chain did not significantly enhance the rate of 3′-pNP/5′-OH ligation. We surmise that other steric or conformational factors serve to constrain the ligation reaction rate (see Discussion). The initial rate of the ligation reaction of Y274F was one-half that of WT topoisomerase and 30% that of Y274A. We surmise from this result that the hydrophobic phenyl moiety imposes a slightly less favorable environment than a polar phenol side chain.

These experiments substantiate a reaction pathway by which topoisomerase catalyzes direct attack of the 5′-OH on the 3′-pNP without forming a covalent enzyme–substrate intermediate.

Catalytic residue Arg223 is required for 3′-pNP/5′-OH ligation

In order to address if the 3′-pNP/5′-OH ligation reaction is catalyzed by the same functional groups on the enzyme that mediate the standard topoisomerase transesterification reactions (cleavage and ligation), we tested the 3′-pNP/5′ OH ligase activity of mutant protein R223A. Alanine substitution at Arg223 elicits a 10^{-5} decrement in the rate of attack of a 5′-OH oligonucleotide on the covalent intermediate. The R223A mutation has no effect on the non-covalent binding of topoisomerase to DNA. Activity is restored when lysine is introduced at this position (41). It was proposed, based on these mutational data, that the side chain of Arg223 of vaccinia topoisomerase makes an essential contact with one phosphate oxygen in the transition state. In the Cre–DNA cocrystal, the side chain of Arg292 (equivalent to Arg223 of vaccinia topoisomerase) makes a monodentate contact to a non-bridging phosphate oxygen in the covalent and non-covalent Cre–DNA complexes (1).

We found that the vaccinia R223A protein was inactive in polynucleotide ligation on the nicked 3′-pNP/5′-OH duplex substrate (Fig. 3). R223A did not accumulate covalent adduct either. We surmise that the mechanism of activation of the scissile phosphate for nucleophilic attack during 3′-pNP/5′-OH strand joining is the same as that of cleavage/religation.

Release of *para*-nitrophenol

Delineation of an enzymatic reaction requires that all products be identified. We have shown above that vaccinia topoisomerase catalyzes ligation of a nick with 3′-pNP and 5′-OH termini. The proposed reaction pathway would be expected to generate free *para*-nitrophenol in molar equivalency to the DNA strand transfer product. Free *para*-nitrophenol is a chromophore that absorbs light in the visual range. Release of *para*-nitrophenol during reaction of 50 µM unlabeled nicked 3′-pNP/5′-OH DNA with 100 µM WT topoisomerase was thereby monitored by an increase in A_{405} over time. The reaction proceeded to an extent that ~60% of the 3′-pNP DNA was converted to free *para*-nitrophenol product during an 18 h reaction (Fig. 5). The single turnover kinetics of *para*-nitrophenol release (Fig. 5) closely paralleled the kinetics of DNA ligation (Fig. 4).

The use of chromogenic substrates has practical implications for real-time assays of topoisomerase catalysis and high-throughput screening for candidate inhibitors. A drawback of *para*-nitrophenol as the chromophore is that relatively high concentrations (>10 µM) are required for spectrophotometric detection (E_{400nm} = 18 300 M^{-1}cm^{-1}), which thereby mandates the use of high initial substrate and enzyme concentrations. This issue may be circumvented by synthesis and testing of additional 3′-modified chromogenic or fluorogenic DNAs that afford enhanced sensitivity in detection of the chromophore/fluorophore leaving groups.

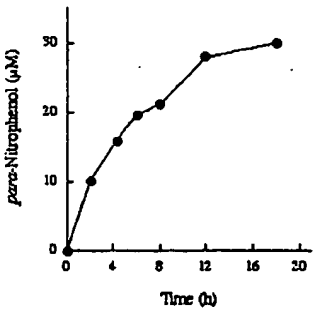

Figure 5. Release of *para*-nitrophenol. Reaction mixtures containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 50 μM of unlabeled nicked 3′-pNP/5′-OH DNA and 100 μM of WT topoisomerase were incubated at 37°C. The reactions were initiated by addition of topoisomerase. Aliquots (10 μl) were withdrawn at the times specified and optical densities at 405 nm were measured using an ultra micro volume cell (5–7 μl working volume) (Amersham Pharmacia, Little Chalfont, UK). The yield of *para*-nitrophenol is plotted as a function of time.

Transesterification of topoisomerase to 3′-pNP in the absence of ligation

We have shown that 3′-pNP/5′-OH ligation need not proceed through a covalent topoisomerase–DNA intermediate. Yet, WT topoisomerase forms a covalent adduct during the ligation reaction. Does this complex represent a potential reaction intermediate in the ligation pathway (albeit not an obligate one) or is the covalent complex exclusively a by-product arising by *ex post facto* cleavage of the ligated duplex DNA product? In essence, the question is whether vaccinia topoisomerase can directly transesterify to the 3′-pNP DNA strand in the absence of prior strand closure. To address this scenario, we replaced the 5′-OH acceptor strand at the nick with a 5′-SH strand of identical sequence. The 5′-SH terminus is at least four orders of magnitude less effective than a 5′-OH as a nucleophile in the standard pathway of DNA strand joining by vaccinia topoisomerase (24).

Parallel reactions of 3′-pNP/5′-SH and 3′-pNP/5′-OH substrates with Y274A and Y274F showed that the 5′-thiol was completely inactive as a nucleophile in attack on the 3′-pNP strand (Fig. 6). (Note that any ligation of the 5′-SH strand would have been detectable because the mutant enzymes cannot recleave the ligation product.) WT topoisomerase again formed both a ligated product and a covalent DNA–protein adduct during its reaction with the 3′-pNP/5′-OH DNA. The instructive finding was that WT topoisomerase reacted with the 3′-pNP/5′-SH nick to yield low levels of the covalent adduct, but formed no ligation product (Fig. 6). We conclude that WT vaccinia topoisomerase can directly transesterify to the 3′-pNP end according to the reaction scheme illustrated in Figure 6. If one assumes that the free *para*-nitrophenol product is released from the covalent complex (a likely prospect given

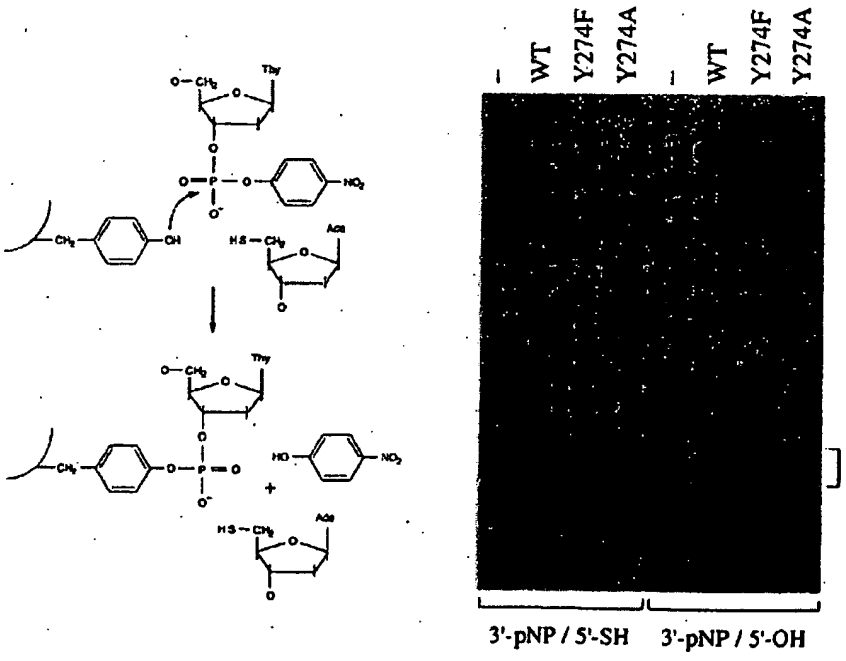

Figure 6. Transesterification to 3′-pNP in the absence of ligation. Reaction mixtures containing (per 20 μl) 50 mM Tris–HCl (pH 7.5), 0.5 pmol of nicked 3′-pNP DNA substrates (containing either 5′-SH or 5′-OH at the nick) and 2.5 pmol of topoisomerase (WT, Y274F or Y274A) were incubated at 37°C for 24 h. The reactions were quenched with SDS and the mixtures were digested with proteinase K. Control reactions lacked topoisomerase (–). The DNA was ethanol-precipitated and analyzed by electrophoresis through a 20% polyacrylamide gel containing 7 M urea in TBE. An autoradiogram of the gel is shown. The covalent DNA–peptide adducts are indicated by the parenthesis on the right. A proposed reaction pathway is illustrated on the left.

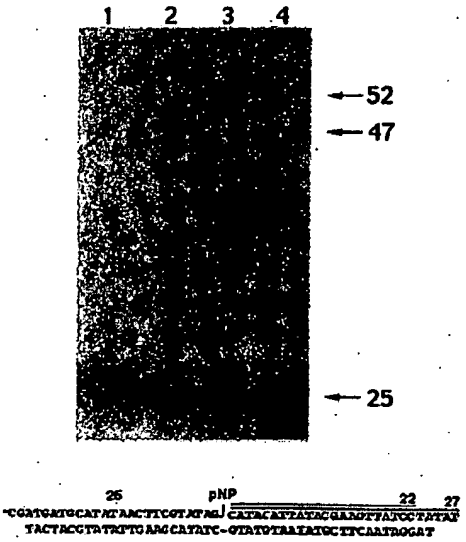

Figure 7. Ligation activity of Cre recombinase. Reaction mixtures containing (per 20 µl) 100 mM Tris–HCl (pH 7.5), 4 mM spermidine, 4 µg/ml BSA, 10% glycerol, 100 nM 3′-pNP/5′-OH or 3′-PO_4/5′-OH DNA substrate as specified and 2.5 µM of Cre recombinase Y324A were incubated at 30°C for 2 h. Recombinase was omitted from control reaction mixtures (lane 1). Reactions analyzed in lanes 2 and 3 were identical except that different 5′-OH oligonucleotides were used to form the *lox* site-containing 3′-pNP/5′-OH substrates depicted at the bottom of the figure; the substrate in lane 2 was prepared with a 22-nt acceptor oligonucleotide whereas the substrate in lane 3 was formed with a 27-nt acceptor stand. Reactions were quenched and the products resolved on a 15% polyacrylamide gel containing 8 M urea in TBE. An autoradiogram of the gel is shown. The positions of the 5′-^{32}P-labeled 25mer 3′-pNP substrate strand and the 47mer or 52mer ligation products are indicated on the right.

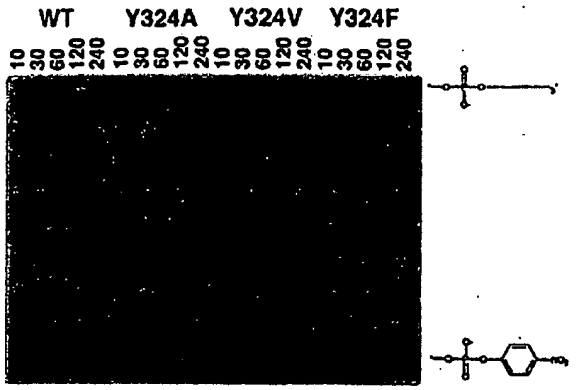

Figure 8. Ligation activity of Cre recombinase does not require the active site nucleophile Tyr324. Reaction mixtures containing (per 40 µl) 100 mM Tris–HCl (pH 7.5), 4 mM spermidine, 4 µg/ml BSA, 10% glycerol, 100 nM 3′-pNP/5′-OH DNA substrate and 2.5 µM Cre recombinase (WT, CreY324A, CreY324V or CreY324F) were incubated at 30°C. The reactions were initiated by addition of recombinase and aliquots (7.5 µl) were withdrawn at the times specified and quenched by the addition of an equal volume of 8 M urea. The samples were analyzed by electrophoresis through a 15% polyacrylamide gel containing 8 M urea in TBE. The resulting autoradiogram is shown. The position of 3′-pNP DNA is shown at the bottom of the autoradiogram and the position of ligated product is illustrated above.

that the topoisomerase releases even a 5′-OH DNA leaving group unless it is annealed to the complementary non-scissile strand), then the observed lower extent of accumulation of covalent adduct on 3′-pNP/5′-SH versus 3′-pNP/5′-OH DNA implies that most of the covalent adduct generated during incubation with 3′-pNP/5′-OH DNA arises via cleavage of the ligated duplex product.

3′-pNP/5′-OH ligation by Cre recombinase

Cre-mediated strand cleavage and ligation reactions occur at specific phosphodiester bonds within a well-defined recombination site, termed a *lox* site. A single *lox* site contains two inverted Cre binding sites; one Cre monomer directs the cleavage and ligation of the 'top' strand and the second Cre monomer directs the cleavage and ligation of the complementary 'bottom' strand (42,43). In order to test whether 3′-pNP DNA could be joined by a tyrosine recombinase, a 25mer 5′-^{32}P-labeled oligonucleotide containing a 3′-pNP moiety was hybridized with two other oligonucleotides to form a singly nicked 'top' strand *lox* site. The resulting nick placed the 25mer 3′-pNP and a 5′-OH 22mer strand at one site of Cre-mediated catalysis (Fig. 7). The *lox* site was incubated in the absence (lane 1) or presence (lane 2) of CreY324A and the reaction products were resolved by PAGE. CreY324A-catalyzed displacement of the nitrophenyl group by the 5′-OH yielded a 47-nt strand transfer product (Fig. 7, lane 2). In order to verify that the ligation product was the result of strand transfer to the 5′-OH oligonucleotide at the nick, a second 3′-pNP/5′-OH *lox* site was constructed with a 5′-OH 27mer acceptor strand at the nick. In this instance, reaction with CreY324A yielded a larger 52mer ligation product (Fig. 7, lane 3). Reaction of CreY324A with a different nicked *lox* site containing 3′-PO_4 and 5′-OH termini at the nick resulted in no detectable strand transfer (Fig. 7, lane 4). The fact that a Cre mutant that lacks a tyrosine nucleophile catalyzed this ligation reaction strongly argues that ligation must result from the direct attack of the 5′-OH from the acceptor oligonucleotide on the 3′-pNP.

As described above, the chemically synthesized 3′-pNP moiety acts as a leaving group in the ligation transesterification reaction. Although the resulting *para*-nitrophenol presumably diffuses away from the active site, the nitrophenyl group must be accommodated within the active site of the enzyme prior to displacement. We therefore tested the ability of different active site mutants that would be predicted to create more space to accommodate the nitrophenyl group to catalyze strand ligation. 5′-End labeled oligonucleotides containing a 3′-pNP moiety were hybridized to form a nicked *lox* site as described above. This pre-activated *lox* site was then incubated with WT Cre, CreY324A, CreY324V and CreY324F and the amount of ligated product was measured as a function of time (Fig. 8). The results demonstrate that the wild-type and all three mutants catalyze ligation at the same rate. Forty percent of the substrate was converted to ligated product after an overnight incubation (data not shown). It is also important to note that covalent Cre–DNA intermediates can be easily detected using 5′-end-labeled phosphorothiolate suicide substrates (21), however no Cre–DNA adducts could be detected when WT

Cre recombinase was incubated with 5′-end-labeled 3′-pNP DNA (data not shown).

DISCUSSION

We have described the synthesis of oligonucleotides containing a 3′-phospho-(*para*-nitrophenyl) moiety. These oligonucleotides (3′-pNP DNA) mimic the 3′-phosphotyrosine adducts formed when type IB topoisomerases and tyrosine recombinases cleave DNA except that the tyrosine analog (*para*-nitrophenyl) is not covalently attached to the enzyme. Vaccinia topoisomerase and Cre recombinase can catalyze the attack of a 5′-OH DNA on 3′-pNP DNA to displace *para*-nitrophenol and ligate the DNA backbone. Mutated versions of vaccinia topoisomerase and Cre that lack the tyrosine nucleophile, and therefore cannot form a covalent intermediate, still catalyze this ligation reaction with kinetics similar to the respective wild-type enzymes. These results demonstrate that 3′-pNP DNA substrates can be used as single-turnover ligation substrates because DNA strand transfer can be assayed in the absence of any competing DNA cleavage reactions.

It is possible to measure the rate of ligation by vaccinia topoisomerase because the enzyme–DNA covalent complex can be recovered in high yield. However, this is not easily accomplished for tyrosine recombinases. During a complete recombination reaction, four phosphodiester bonds are cleaved and four new DNA phosphodiester bonds are formed. These reactions occur in a complex of at least four different recombinase monomers. It is therefore very difficult to pre-form functional enzyme–DNA adducts to assay DNA strand ligation. In addition, because the cleavage/ligation transesterification reactions are isoenergenic and occur in a synaptic complex, ligation intermediates that form may not be detected because competing cleavage reactions may reform starting material, or another reaction product along the reaction pathway. 3′-pNP DNA substrates solve these problems because the substrates are chemically synthesized and can be introduced into the reaction as 'nicked' substrates. Most importantly, 3′-pNP/5′-OH nicked substrates allow individual ligation reaction products to accumulate because subsequent tyrosine-mediated cleavage reactions are not possible if the ligation is performed by a mutant enzyme that lacks a tyrosine nucleophile.

There are two caveats to this conclusion. First, it is possible that the released *para*-nitrophenol can re-attack the ligated DNA product thereby allowing subsequent cleavage reactions by regenerating an activated phosphodiester. Indeed, Lee and Jayaram have demonstrated that Flp recombinase tyrosine mutants can use *para*-nitrophenol as an exogenous nucleophile in DNA cleavage reactions (29). However, these reactions are inefficient and require free concentrations of 1–10 mM *para*-nitrophenol (29). In the experiments described above, the highest concentration of free *para*-nitrophenol is 0.00004 mM. It is also important to note that, unlike Cre and vaccinia topoisomerase, Flp-catalyzed DNA cleavage occurs *in trans*; the tyrosine residue used to cleave the phosphodiester backbone comes from an Flp protomer bound to a second partner DNA substrate. One would therefore predict that Flp, unlike Cre or vaccinia toposiomerase, would be able to use exogenous *trans*-cleaving nucleophiles. It has been clearly demonstrated that vaccinia topoisomerase cannot use exogenous nucleophiles for DNA cleavage reactions (20).

Second, it is theoretically possible that when a tyrosine mutant is used to catalyze ligation, a different enzyme-bound nucleophile attacks the 3′-pNP DNA or can attack the ligated DNA to form an activated phosphodiester for subsequent reactions. Many results argue against such an aberrant side reaction. For example, no enzyme–DNA adducts could be detected for the tyrosine mutants described using 5′-bridging phosphorothiolate (21) cleavage suicide substrates (data not shown). In addition, covalent adducts were easily detected when WT vaccinia topoisomerase was used but no covalent adducts could be detected when a variety of different tyrosine mutants were used (Fig. 3). The same result was even obtained when ligation was blocked by replacing the 5′-OH with a 5′-SH at the nick (Fig. 6). If a previously unidentified enzyme-bound nucleophile was attacking the 3′-pNP linkage or ligated DNA product, a covalent topoisomerase–DNA adduct would be expected to accumulate under this circumstance. In addition, the fact that vaccinia topoisomerase R223A exhibited no ligation activity (Fig. 3) argues that the 3′-pNP/5′-OH ligation reaction is catalyzed by the same functional groups on the enzyme that mediate the standard topoisomerase transesterification reactions. Finally, because the rate of Cre catalyzed 3′-pNP/5′-OH ligation is the same when wild-type or tyrosine mutants are used, it is unlikely that a previously unidentified side reaction occurs specifically in the tyrosine-deficient reaction.

It is worth emphasizing that the rate of vaccinia topoisomerase-catalyzed 3′-pNP/5′-OH ligation (5×10^{-5} s^{-1}) is at least four orders of magnitude slower than the rate constant for topoisomerase-catalyzed attack of 5′-OH DNA on the covalent DNA-(3′-phosphotyrosyl)–enzyme intermediate ($k_{rel} \sim 1$ s^{-1}). As discussed above, it is not yet possible to make this same comparison for any tyrosine recombinase because these systems are not kinetically well-defined and recombinase–DNA complexes cannot be reliably assayed. The simplest explanation for the observed slow rate of 3′-pNP/5′-OH ligation is that steric constraints within the active site prevent the *para*-nitrophenyl group from achieving an optimal fit relative to the other catalytic side chains and/or an optimal apical orientation of the *para*-nitrophenyl leaving group relative to the attacking 5′-OH DNA nucleophile. Another possible explanation is that the enzyme bound 3′-pNP substrate must go through a slow conformational transition to place the *para*-nitrophenyl group in the active site (e.g., if the *para*-nitrophenyl ring intercalates into the nick instead of projecting out of the DNA helix). This conformational transition would not be necessary in the normal reaction because the positioning is certainly determined by the covalent attachment of the tyrosine to the enzyme.

The *para*-nitrophenyl group is relatively small and we entertained the hypothesis that mutations that would better accommodate 3′-pNP within the active site (topoisomerase Y274A and CreY324A) might improve the rate of 3′-pNP ligation. Clearly this is not the case. We cannot rule out the possibility that the smaller side chains in lieu of tyrosine cause unexpected distortions in the active site that inhibit catalysis *per se* and thereby mask salutary effects on 3′-pNP binding.

The DNA 3′-pNP strand transfer reaction described herein differs from the usual topoisomerase/recombinase-mediated transesterifications in that it occurs at a pre-existing nick. A cardinal feature of enzymatic nick-joining reactions is the covalent modification of one side of the nick to activate it for

attack by the opposing hydroxyl. Whereas vaccinia topoisomerase ligated very slowly when the activating moiety at the nick is a 3′-pNP; it is much more active when the nick is activated by a 2′,3′-cyclic phosphodiester (17). For example, sealing of a 2′,3′-cyclic phosphate (2′,3′>p)/5′-OH nick proceeds to the reaction endpoint in <10 min, compared to ~18 h for a 3′-pNP/5-OH nick. Although the active site tyrosine is not essential for ligation of a nick with a 2′,3′>p, its presence does accelerate the reaction rate. Catalysis through the covalent intermediate is ~20-fold faster than direct attack of the 5′-OH on the 2′,3′>p (17). This is in contrast to the situation described here for 3′-pNP activated substrates, in which the tyrosine does not contribute to the observed ligation rate. A simple explanation for why the 2′,3′>p is a much better substrate for ligation by topoisomerase is that there is little or no steric clash of a cyclic phosphate in the active site.

The fact that vaccinia topoisomerase can catalyze the release of *para*-nitrophenol (Fig. 5) from 3′-pNP DNA has important implications for developing high throughput assays for new anti-cancer drugs. This conclusion follows from the observation that eukaryotic topoisomerases have been demonstrated to be valuable targets for anti-cancer drugs (44). A structurally diverse set of anti-cancer compounds including camptothecin, some minor groove binding ligands, some indolocarbazole derivatives and protoberberine alkaloids, appear to function by specifically blocking or slowing the rate of religation following strand cleavage by human topoisomerase I. As a result, these topoisomerase poisons stabilize the normally transient 3′-phosphotyrosyl covalent complex and turn the topoisomerase into a DNA damaging agent. Clearly, new drugs that also block religation would be expected to slow the release of *para*-nitrophenol from 3′-pNP DNA. Because *para*-nitrophenol can be assayed colorimetrically, a large number of individual compounds or mixtures can be quickly screened.

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Brief Report

Herpes Simplex Virus Type 1 DNA Amplified as Bacterial Artificial Chromosome in *Escherichia coli:* Rescue of Replication-Competent Virus Progeny and Packaging of Amplicon Vectors

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ABSTRACT

Herpes simplex virus type 1 (HSV-1)-based amplicon vectors contain only ~ 1% of the 152-kb HSV-1 genome, and consequently, replication and packaging into virions depends on helper functions. These helper functions have been provided conventionally by a helper virus, usually a replication-defective mutant of HSV-1, or more recently, by a set of five cosmids that overlap and represent the genome of HSV-1 deleted for DNA cleavage/packaging signals (*pac*). In the absence of *pac* signals, potential HSV-1 genomes that are reconstituted from the cosmids via homologous recombination are not packageable. The resulting amplicon stocks are, therefore, virtually free of contaminating helper virus. To simplify this packing system, the HSV-1 genome was cloned and maintained stably as a single-copy, F plasmid-based bacterial artificial chromosome in *E. coli*. Such a plasmid containing the HSV-1 genome deleted for the *pac* signals (fHSVΔ pac) did not generate replication-competent progeny virus on transfection into mammalian cells, but rather, it was able to support the packaging of cotransfected amplicon DNA that contained a functional *pac* signal. The resulting amplicon vector stocks had titers of up to 10^7 transducing units per milliliter of culture medium and efficiently transduced neural cells in the rat brain, as well as hepatocytes in the rat. The capacity of generating infectious and replication-competent HSV-1 progeny following transfection into mammalian cells was restored after insertion of a *pac* signal into fHSVΔpac.

INTRODUCTION

VECTORS BASED ON herpes simplex virus type 1 (HSV-1) are potent gene delivery vehicles because they have large transgene capacities and can efficiently infect many different cell types (Breakefield *et al.*, 1995; Glorioso *et al.*, 1995). There are two types of HSV-1 vector systems: recombinant and amplicon. Recombinant HSV-1 vectors are created by inserting genes of interest directly into the 152-kb virus genome, thereby deleting one or more of the ~80 virus genes to reduce cytotoxicity. In contrast, HSV-1 amplicons are bacterial plasmids that contain (1) a transgene cassette with the gene of interest, (2) sequences that allow plasmid propagation in *Escherichia coli*, such as the origin of DNA replication *colE1* and the ampicillin resistance gene, and (3) noncoding elements of the HSV-1 genome, in particular an origin of DNA replication (*ori*) and a DNA cleavage/packaging signal (*pac*, to support replication and subsequent packaging of the amplicon DNA into

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virions in the presence of helper functions (Spaete and Frenkel, 1982). Because HSV-1 encodes many toxic functions, efforts to improve the amplicon system have been targeted primarily at reducing the risk associated with the helper virus. First, replication-competent HSV-1, initially used as helper virus, was replaced by a temperature-sensitive (ts) mutant of HSV-1 (HSV-1 tsK; Preston, 1979). This mutant encodes a temperature-sensitive form of the essential HSV-1 infected cell protein 4 (ICP4), allowing HSV-1 replication to proceed at 31°C, but not at 37°C. Amplicons packaged at 31°C in the presence of HSV-1 tsK were successfully used to transfer the *E. coli lacZ* gene into primary cultures of rat neural cells (Geller and Breakefield, 1988). Because the infection was performed at 37°C, the lytic cycle of the HSV-1 tsK helper virus present in the vector stock was blocked and cell damage was limited. Although replication of HSV-1 tsK was inhibited at the restrictive temperature, the expression of other viral genes caused cytopathic effects. Moreover, reversion to wild-type (wt) HSV-1 occurred at a relatively high frequency owing to remaining functionality and reversion of the point mutation in tsICP4. To counter these problems, replication-defective mutants of HSV-1 were introduced as helper viruses (Geller *et al.*, 1990; Lim *et al.*, 1996). These mutants carry deletions in genes that are essential for virus replication, but they can support amplicon packaging in cells that complement the missing functions. In general, deletion-mutant packaging systems produce relatively high amplicon vector titers (10^6–10^7 transducing units [TU] per milliliter), a ratio of transducing vector units to helper virus of up to 1, and low levels of revertants with wt HSV-1 phenotype ($<10^{-7}$ plaque-forming units, [PFU] per milliliter; Lim *et al.*, 1996). However, many problems associated with the presence of helper virus in amplicon stocks still remained, including (1) pronounced cytopathic effects and immune responses caused by gene expression from the helper virus, (2) interactions between the helper virus and endogenous viruses, and (3) reversion to wt HSV-1. The development of a helper virus-free packaging system eliminated many of these problems (Fraefel *et al.*, 1996). This system utilizes transient cotransfection of amplicon DNA with a set of five cosmids that overlap and represent the entire HSV-1 genome, but which were mutated to remove the *pac* signals. Cunningham and Davison (1993) demonstrated previously that after transfection into cells, an overlapping HSV-1 cosmid set can produce infectious virus progeny. By deleting the *pac* signals, virus genomes that are potentially reconstituted, via homologous recombination, are not packageable, but can still provide all the helper functions required for the replication and packaging of cotransfected amplicon DNA. The resulting vector stocks are virtually free of detectable helper virus and have titers of 10^6–10^7 TU/ml of culture medium. Because of minimal sequence homology between the cosmids and the amplicon DNA (*ori*$_S$; 0.2–1 kb), the formation of a packageable and replication-competent HSV-1 genome is possible but requires six recombination events, and is therefore rare. Amplicon vectors stocks, produced by using the helper virus-free packaging system, can efficiently transduce many different cell types, including neural cells and hepatocytes in culture and *in vivo*, while causing minimal to no cytopathic effects (Fraefel *et al.*, 1996, 1997, 1998; Aboody-Guterman *et al.*, 1997; Johnston *et al.*, 1997).

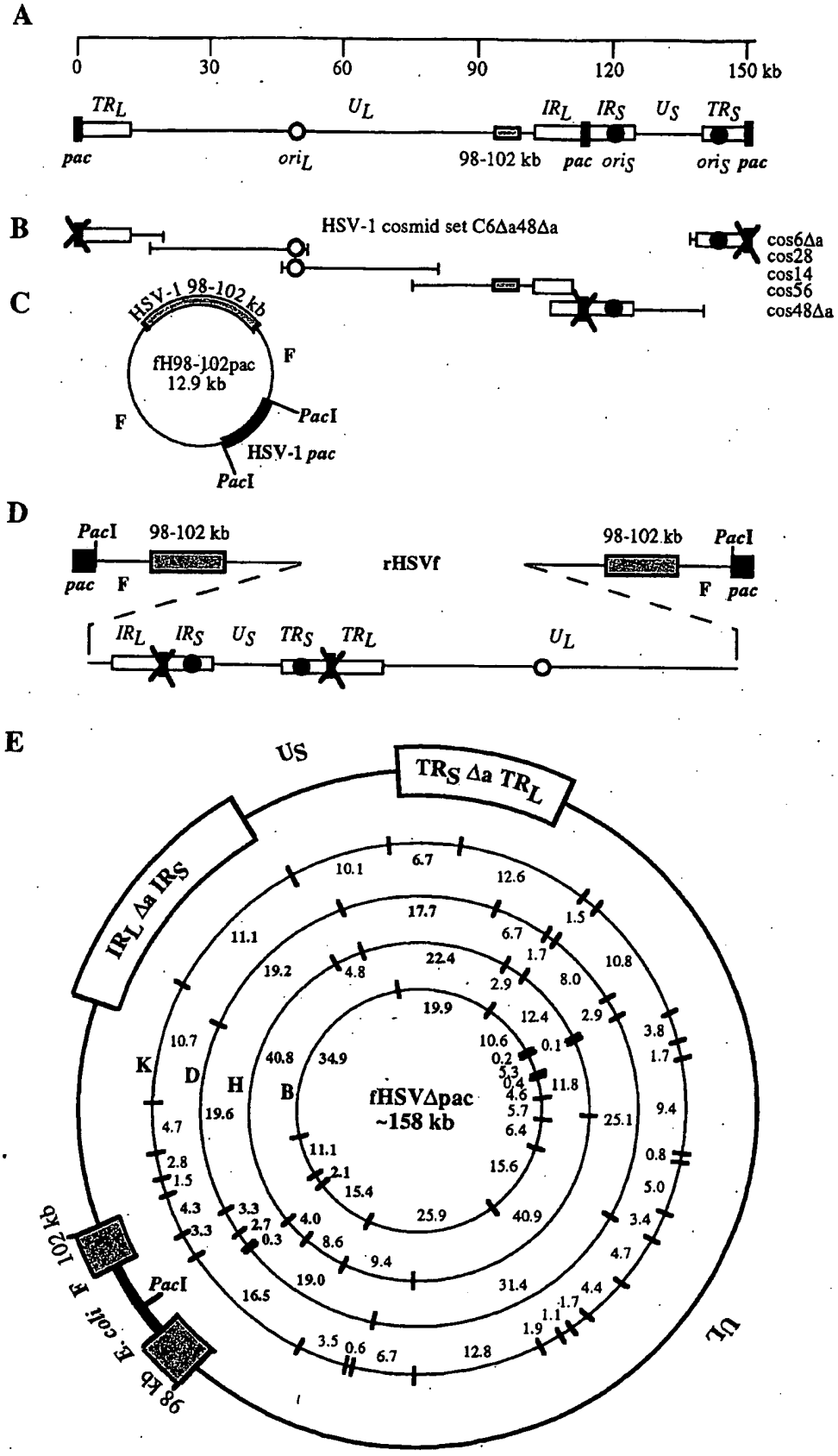
Although the production of vector particles requires the cells to be simultaneously cotransfected with all five clones of the cosmid set and the amplicon DNA, the titers of the resulting amplicon stocks are surprisingly high and comparable to those achieved with the helper virus-dependent systems. Nevertheless, by reducing the number of clones representing the HSV-1 genome, ideally to a single clone, it may be possible to further increase the packaging efficiency. To investigate this possibility, we cloned the 152-kb HSV-1 genome, with or without a *pac* signal, as a bacterial artificial chromosome (BAC) in *E. coli*.

MATERIALS AND METHODS

Cell culture

African green monkey kidney cells (VERO76, ECACC) and human embryonic kidney cells (293; American Type Culture Collection [ATCC], Rockville, MD) were propagated in Dulbecco's modified minimal essential medium (DMEM) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (P/S) and 10% fetal bovine serum (FBS). 2-2 cells (kindly provided by R. Sandri-Goldin, University of California, Irvine, CA; Smith *et al.*, 1992), which are VERO-derived cells that constitutively express the HSV-1 ICP27 protein, were cultivated in DMEM containing P/S, 10% FBS, and G418 (Geneticin, 500 μg/ml; GIBCO-BRL, Gaithersburg, MD).

FIG. 1. Cloning of the HSV-1 genome as a bacterial artificial chromosome in *E. coli*. (A) The HSV-1 genome (~152 kb) is composed of unique long (U_L) and unique short (U_S) segments (horizontal lines), which are flanked by inverted repeats (open rectangles): IR_S, internal repeat of the short segment; TR_S, terminal repeat of the short segment; IR_L, internal repeat of the long segment; TR_L, terminal repeat of the long segment. The origins of DNA replication, ori_S (solid circle) and ori_L (open circle), and the DNA cleavage/packaging signals, *pac* (solid rectangles), are shown, (B) Cosmid set C6Δa48Δa represents the HSV-1 genome deleted for the *pac* signals (X) and includes cos6Δa, cos14, cos28, cos49Δa, and cos56.) (C) Transfer plasmid fH98-102pac, which is based on the single-copy F-plasmid from *E. coli*, contains (1) sequences between nucelotides 98,968 and 102,732 of the HSV-1 genome to facilitate homologous recombination with HSV-1 DNA from cosmid set C6Δa48Δa, and (2) the HSV-1 *pac* signal flanked by recognition sites for restriction endonuclease *Pac*I. (D) Hypothetical structure of replication-competent virus (rHSVf) generated in 2-2 cells on cotransfection of transfer plasmid fH98-102pac and the five clones of cosmid set C6Δa48Δa. DNA isolated from rHSVf was digested with *Pac*I, and a fragment of ~160 kb was isolated, self-ligated, and then electroporated into *E. coli* DH10B. (E) Predicted structure of a bacterial artificial chromosome carrying the HSV-1 genome deleted for the *pac* signals (fHSVΔ pac). The sites for restriction endonucleases *Kpn*I(K), *Dra*I(D), *Hin*dIII(H), *Bf*LII(B), and the resulting fragment sizes are indicated. Numbers in boldface represent fragment sizes that differ from the original HSV-1 fragments because of the deletion in the *pac* signals or the insertion of the transfer plasmid.


A
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IRL IRS US TRS TRL
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TRS Δa TRL
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98 kb E. coli 102 kb
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K
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Construction of HSV-1 transfer plasmid fH98-102pac

To construct pBXPBPX, plasmid pBsSK+ (Stratagene, La Jolla, CA) was digested with *Xho*I and ligated to the synthetic oligonucleotide duplex 5′ tcgagggcccttaattaagatcttaattaagggccc 3′, as a method to substitute the pBsSK+ *Xho*I site with the polylinker *Xho*I–*Pac*I–*Bg*LII–*Pac*I–*Xho*I. Plasmid pHSVPrPUC (kindly provided by H. Federoff, University of Rochester, NY) was digested with *Xho*II, and the 1.4-kb fragment, which contains the HSV-1 DNA cleavage/packaging signal (*pac*), was inserted into the *Bg*LII site of pBXPBPX. The resulting plasmid pBXPpacPX was digested with *Xho*I, and the 1.4-kb fragment was inserted into the unique *Xho*I site of pBeloBAC11 (Research Genetics, Huntsville, AL), forming pBeloBACpac. An ~4-kb *Hin*cII fragment (nucleotides 98,742–102,732 of the HSV-1 genome; McGeoch *et al.*, 1988) was isolated from cos56 of HSV-1 cosmid set C (kindly provided by C. Cunningham and A.J. Davison, MRC Virology Unit, Glasgow, UK; Cunningham and Davison, 1993) and inserted first into the *Eco*RV site of pBsSK+. From the resulting subclone, pBH98-102 an ~4-kb *Bam*HI fragment (nucleotides 98,968–102,732 of the HSV-1 genome) was isolated and inserted into the unique *Bam*HI site of pBeloBACpac. The resulting transfer plasmid, fH98-102pac, was used to target integration of (1) the HSV-1 *pac* signal flanked by *Pac*I restriction sites, and (2) *E. coli* F factor-derived sequences into the HSV-1 genome between nucleotides 98,968 and 102,732 (Fig. 1C).

Construction of rHSVf

The five cosmids of set C6Δa48Δa (Fig. 1B; Cunningham and Davison, 1993; Frafel *et al.*, 1996) were digested with *Pac*I to excise the HSV-1 inserts and purified by phenol extraction. 2-2 cells were plated at a density of 1×10^6 cells per 60-mm-diameter tissue culture dish. The following day the cells were transfected with 0.5 μg of fH98-102pac DNA and 0.4 μg of each cosmid DNA by the LipofectAMINE procedure according to instructions provided by the manufacturer (GIBCO-BRL). Three days after transfection, the cells were scraped into the medium, the suspension was frozen and thawed three times, cell debris was removed by centrifugation (10 min, 1400 × *g*), and titers (PFU/ml) of replication-competent virus (rHSVf, see Fig. 1D) were determined by standard plaque assays on VERO cells.

Construction of fHSVΔpac and fHSVpac+

rHSVf (Fig. 1D) was amplified over three passages on VERO cells. When cytopathic effect (CPE) was complete after the third passage, the cells (from twelve 175-mm tissue culture flasks) were scraped into the medium, the suspension (240 ml) was frozen and thawed three times, and cell debris was removed by centrifugation (10 min, 1400 × *g*). After centrifugation for 1 hr at 28,000 × *g* and 4°C, the supernatant was discarded, and the virus pellet was resuspended in 80 ml of balanced salt solution (BSS; 137 m*M* NaCl, 5.4 m*M* KCl, 10 m*M* Tris-HCl [pH 7.6]). After centrifugation for 30 min at 28,000 × *g* and 4°C, the pellet was resuspended in 5.5 ml of TE buffer (10 m*M* Tris-HCl, 10 m*M* EDTA [pH 7.5]) and 150 μl of a 20-mg/ml solution of proteinase K. The suspension was incubated for 1 hr at 37°C, and after the addition of 300 μl of a 10% sodium dodecyl sulfate (SDS) solution, further incubated overnight at 37°C. After phenol–chloroform extraction and ethanol precipitation, virus DNA was resuspended in TE buffer, digested completely with *Pac*I restriction endonuclease, and then fractionated by electrophoresis through 0.4% low-melt agarose (GIBCO-BRL) at 40 V overnight. A band corresponding to ~160 kb was excised from the gel and treated with β-agarase I (New England Bio-Labs, Beverly, MA), and the purified DNA was self-ligated with T4 DNA ligase. The ligation products were electroporated into electorcompetent *E. coli* DH10B cells according to instructions provided by the supplier (GIBCO-BRL). Miniprep DNA of several colonies that appeared on LB plates containing a 12.5-μg/ml concentration of chloramphenicol (LB^{CM}) was prepared by alkaline lysis, and clones that contained the HSV-1 genome with *pac* signals deleted (fHSVΔpac) were characterized by restriction enzyme analysis (Figs. 1E and 2). To construct fHSVpac+, fHSVΔpac DNA was digested with *Pac*I, treated with alkaline phosphatase to prevent religation, and then ligated to the 1.4-kb *Pac*I fragment isolated from pBXPpacPX

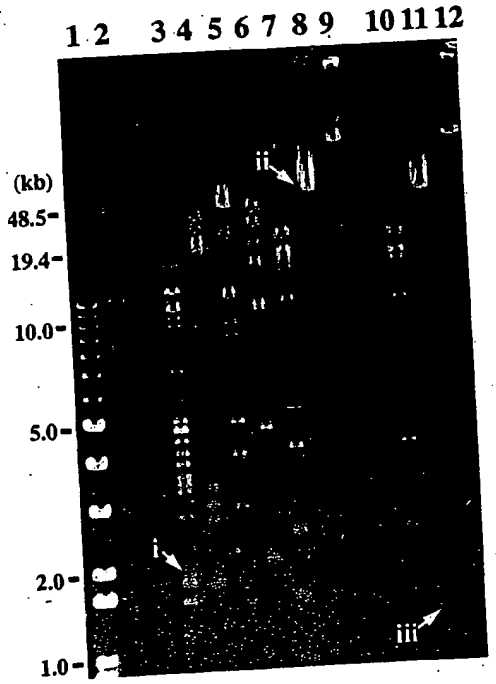

FIG. 2. Analytic agarose gel showing restriction endonuclease patterns of BAC DNA. The reaction mixtures were loaded on a 0.4% agarose gel, and the fragments were separated overnight at 40 V in TAE electrophoresis buffer and stained with ethidium bromide. Lane 1, 1-kb Plus DNA ladder (GIBCO-BRL); lane 2, high molecular weight DNA standard (GIBCO-BRL); lanes 3–9, fHSVΔpac DNA digested with *Kpn*I, *Dra*I, *Hin*dIII, *Bg*LII, *Eco*RI, or *Pac*I, or undigested. The 1.9-kb *Kpn*I fragment containing HSV-1 ori_L (arrow i, lane 3), and the 158-kb *Pac*I fragment (linearized fHSVΔpac; arrow ii, lane 8) are indicated. Lanes 10–12, fHSVpac+ DNA digested with *Eco*RI or *Pac*I, or undigested. The 1.4-kb *Pac*I fragment containing the HSV-1 *pac* signal is indicated (arrow iii, lane 11). To provide adequate contrast for the fragments with different sizes in the entire gel, this gel image is composed of three pictures with different exposures.

(see above). After electroporation of the ligation products into *E. coli* DH10B and selection of colonies on LB^{CM} plates, clones that contained the HSV-1 *pac* signal were characterized by restriction enzyme analysis (Fig. 2).

Generation of virus plaques from fHSVpac+

Large amounts of BAC DNA were prepared from 1 l cultures grown in LB^{CM}. Plasmid DNA was extracted by alkaline lysis and purified first over Tip-500 columns, as described by the manufacturer (Qiagen, Chatsworth, CA), and then by cesium chloride equilibrium centrifugation (Sambrook *et al.*, 1989). BAC DNA (fHSVΔpac of fHSVpac+) or HSV-1 cosmid DNA (set C or set C6Δa48Δa; Cunningham and Davison, 1993; Fraefel *et al.*, 1996) was transfected into 2-2 cells by using LipofectAMINE as described above, except that after transfection, the cultures were incubated in DMEM that contained 2% FBS and 0.8% carboxymethyl cellulose (BDH Chemicals, Poole, UK). After 2 days, the cultures were stained with crystal violet to visualize plaques.

Packaging of amplicon DNA into HSV-1 virions and titration of vector stocks

2-2 cells were cotransfected with 0.6 μg of pHSVGFP amplicon DNA (expresses the gene for green fluorescent protein, GFP; Aboody-Guterman *et al.*, 1997) and 2 μg of fHSVΔpac DNA by the LipofectAMINE procedure, as described above. After 3 days, the cells were scraped into the medium, the suspension was frozen and thawed three times, and cell debris was removed by centrifugation (10 min, 1400 × *g*). To determine vector titers (transducing units [TU] per milliliter), 293 cells were infected and, 24 hr later, green fluorescent cells were counted using a fluorescence microscope equipped with a filter set for detection of enhanced GFP. Titers of replication-competent virus (plaque-forming units per milliliter) were determined by standard plaque assays on VERO cells. For gene transfer experiments into the rat brain and liver, pHSVGFP vector stocks were filtered (0.8-μg pore size cellulose nitrate membrane filters; Nalgene, Rochester, NY) and then centrifuged for 2.5 hr at 100,000 × *g* through 25% sucrose (in phosphate-buffered saline [PBS]. The pellet was resuspended in Hanks' BSS and the titer of pHSVGFP vector was determined on 293 cells, as described above.

Gene transfer into rat brain and rat liver

Animal studies were performed in accordance with guidelines issued by the Massachusetts General Hospital Subcommittee on Animal Care. Care of experimental animals, and inoculations of HSV-1 amplicon vectors, were carried out in approved BL-2 rooms. Male CD Fisher rate (300 g; Charles River Laboratories, Wilmington, MA) were anesthetized with ketamine (12.5 mg) and xylazine (2.5 mg). Stocks of pHSVGFP vector were either delivered (7.5×10^5 TU in 10 μl) under stereotaxic guidance into the right hippocampus (AP −3.5, R 2.0, V −3.5; Paxinos and Watson, 1986), or injected (7.5×10^6 TU in 100 μl) under the perisplanchnic membrane into the caudal liver lobe. After 3 days, the animals were anesthetized and perfused with PBS followed by 4% paraformaldehyde in PBS. Brains and livers were postfixed overnight in 4% paraformaldehyde in PBS, cryoprotected for 2 days with 30% sucrose in PBS, and cut into 20-μm cryostat sections. The sections were mounted on glass slides, coverslipped by using Gel/Mount medium (Biomeda, Foster City, CA), and examined with a confocal laser microscope (BioRad [Hercules, CA] MRC-1024).

RESULTS AND DISCUSSION

HSV-1 cosmid sets have been employed to facilitate the construction of recombinant viruses and, after deletion of the HSV-1 *pac* signals, to mediate the helper virus-free packaging of HSV-1 amplicons into virions (Cunningham and Davison, 1993; Fraefel *et al.*, 1996). Although the packaging of amplicons requires that the cells be cotransfected by all five clones of the cosmid set and the amplicon DNA, the resulting vector titers are relatively high, with 10^6–10^7 TU/ml. The goal of this study was to assess whether a single clone, in particular a bacterial artificial chromosome (BAC), that contains the HSV-1 genome deleted for the *pac* signals could further increase the titers of amplicon vector stocks. BACs are based on the single-copy F plasmid of *E. coli* and have been demonstrated previously to stably maintain human genomic DNA of >300 kb, and genomes of large DNA viruses, including those of baculovirus and murine cytomegalovirus (Shizuya *et al.*, 1992; Luckow *et al.*, 1993; Messerle *et al.*, 1997).

Cloning of the HSV-1 genome as a bacterial artificial chromosome

Figure 1 illustrates the strategy used to clone the HSV-1 genome as a BAC in *E. coli*. First, transfer plasmid fH98-102pac was designed to have the following features: (1) Sequences that mediate homologous recombination with HSV-1 DNA between nucleotides 98,968 and 102,732 (McGeoch *et al.*, 1998), (2) the HSV-1 DNA cleavage/packaging signal (*pac*) flanked by recognition sites for restriction endonuclease *Pac*I, and (3) *E. coli* F factor-derived sequences, which allow amplification of the clone as a single-copy plasmid in bacteria (Willetts and Skurray, 1987; Fig. 1C). Transfer plasmid fH98-102pac was cotransfected with five cosmids that represent the entire HSV-1 genome as overlapping clones, but that were previously mutated to remove the *pac* signals (cosmid set C6Δa48Δa; Fig. 1B; Cunningham and Davison, 1993; Fraefel *et al.*, 1996). Three days after transfection, progeny virus was harvested and demonstrated to contain replication-competent virus (rHSVf) by standard plaque assay on VERO cells (1.25×10^4 PFU/ml). The cosmid clones can form a complete replication-competent virus genome via homologous recombination between the overlapping sequences. However, these virus genomes are only packageable if a *pac* signal is acquired through integration of the transfer plasmid fH98-102pac via a single cross-over within the ~4 kb of sequence homology (nucleotides 98,968–102,732 of the HSV-1 genome). After amplification of this original stock of rHSVf, virion DNA was isolated and digested with *Pac*I (which does not cut wild-type HSV-1 DNA) to remove the *pac* signal (Fig. 1D). From this digestion, a DNA fragment of approximately 160 kb was isolated and self-ligated with T4 DNA ligase. The ligation product was

TABLE 1. PLAQUE-FORMATION AFTER TRANSFECTION OF *Escherichia coli* CLONES REPRESENTING THE HSV-1 GENOME[a]

E. coli *clone*	*Amount of transfected DNA (μg)*	*Number of plaques (2 days pt)*[b]
fHSVΔpac	1.5	0 ± 0
cos set C6Δa48Δa	1.5	0 ± 0
fHSVpac+	0.2	873 ± 164
	0.5	CPE[c]
	1.5	CPE
cos set C	1.5	20 ± 6

[a]Cells were transfected as described in text.
[b]Numbers represent the mean ± SD.
[c]A general CPE was observed. Plaques were too numerous to be counted.
Abbreviation: pt, Posttransfection.

then electroporated into electrocompetent *E. coli* DH10B cells. Of the colonies appearing on selective plates, several clones that contained large inserts were identified, and one clone, designated fHSVΔpac, was further characterized by restriction enzyme analysis. Digestion with various restriction endonucleases, including *Kpn*I, *Dra*I, *Hin*dIII, *Bg*LII, *Eco*RI, and *Pac*I, identified all bands predicted from the published HSV-1 sequence, including the ~1.9-kb *Kpn*I band that frequently suffers ori_L-associated deletions on cloning in bacteria (Polvino-Bodnar *et al.*, 1987; McGeoch *et al.*, 1988; Figs. 1E and 2). Moreover, this pattern of restriction fragments was entirely conserved, even after three consecutive colony purifications, thereby confirming the structural stability of large single-copy plasmids (not shown).

Generation of infectious HSV-1 from fHSVpac+ DNA amplified in Escherichia coli

Confirming our hypothesis, fHSVΔpac did not generate HSV-1 progeny virus on transfection into cells in culture (Table 1). To assess whether the capability of generating replication-competent virus progeny can be restored by inclusion of the *pac* element in the BAC clone, fHSVpac+ was constructed by inserting the *pac* signal into the unique *Pac*I site of fHSVΔpac (see Fig. 2). As shown in Table 1, transfection of 0.2 μg of fHSVpac+ DNA gave rise to more than 800 plaques at 2 days after transfection. Higher amounts of DNA produced a com-

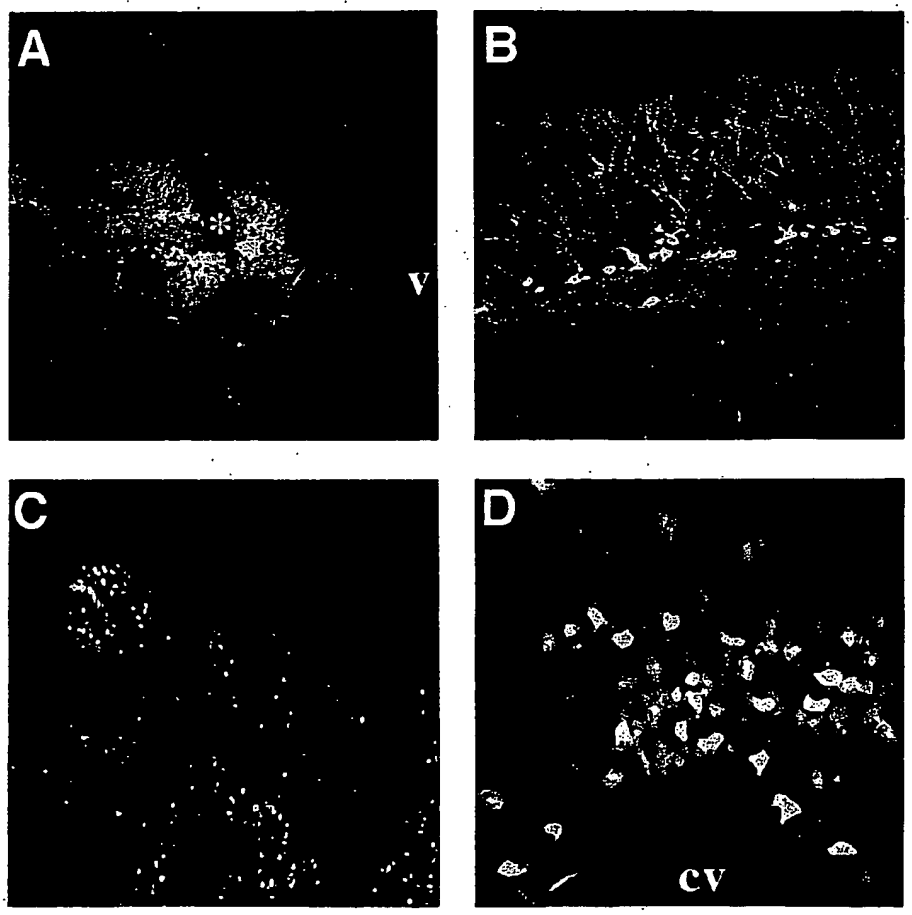

FIG. 3. Photomicrogarphs showing GFP-positive cells on day 4 following injection of pHSVGFP amplicon vector into hippocampus (7.5×10^5 TU, A and B) or liver (7.5×10^6 Tu, C and D). Needle track (*), third ventricle (v), and central vein (cv) are indicated. Original magnification: (A and C) ×5; (B and D) ×20.

plete CPE in the same time period. In contrast, 1.5 μg of DNA from cosmid set C resulted only in an average of 20 plaques, a number similar to that reported by Cunningham and Davison (1993).

fHSVΔpac-mediated packaging of amplicons into HSV-1 virions

Although fHSVΔpac alone did not generate replication-competent virus progeny, this clone could efficiently package cotransfected pHSVGFP amplicon DNA into HSV-1 virions and generate vector stocks with titers of $8.6 \times 10^6 \pm 4.0 \times 10^6$ TU/ml (mean ± SD; $n = 3$ experiments). Although the efficiency of plaque formation by fHSVpac+ was more than 400-fold higher than that of cosmid set C (see Table 1), the amplicon vector titers realized with fHSVΔpac showed only a 2-fold increase compared with cosmid set C6Δa48Δa. This may be due to the limited replication capacity of the amplicon, because preliminary studies indicate that somewhat higher vector titers (twofold) can be achieved with amplicons containing the simian virus 40 (SV40) origin of DNA replication in cells transfected with the SV40 T antigen (T.A. Stavropoulos and C.A. Strathdee, personal communication). Because of a sequence homology between fHSVΔpac and pHSVGFP (~1 kb containing *ori*$_S$ and IE4/5 promoter; Aboody-Guterman *et al.*, 1997) that can support the generation of a packageable HSV-1 genome via a single homologous recombination event, the vector stocks were expected to be heavily contaminated by replication-competent virus. However, contamination by such viruses was surprisingly low, with 59 ± 52 PFU/ml (mean ± SD; $n = 3$ experiments), resulting in a ratio of transducing vector units to replication-competent virus of $>1.5 \times 10^5$. Consequently, the injection of 7.5×10^5 TU of pHSVGFP vector (along with potentially five replication-competent virus particles) into the hippocampus of rats resulted in thousands of GFP-positive cells in this area with minimal cytotoxic effects on day 4 after injection (Fig. 3A and B). There was some tissue necrosis, bleeding, and influx of immune cells at the injection site, as is typical of replication-defective adenovirus and HSV-1 vectors. The injection of 7.5×10^6 TU of pHSVGFP vector into the rat liver resulted also in a high percentage of GFP-positive hepatocytes with the minimal cytopathic changes (Fig. 3C and D). The extent of delivery with these BAC-packaged amplicons appeared similar to that of helper virus-packaged amplicons.

As a possible explanation for the low amount of contamination with replication-competent progeny virus, a DNA molecule generated via a single crossover between pHSVGFP and fHSVΔpac would be larger than 165 kb and may not be packaged as efficiently as a 152-kb molecule (size of the standard HSV-1 genome), even in the presence of a functional *pac* signal. However, further developments should be targeted at eliminating any replication-competent progeny virus. The possibility to generate infectious progeny from fHSVΔpac through rescue with the *pac* signal offer an efficient way of manipulating the large HSV-1 genome for the targeted design of improved HSV-1 vector systems, both recombinant and amplicon. It permits the isolation of recombinant HSV-1 without the need for selection via homologous recombination directly in mammalian cells or in bacteria. If flanked by recognition sites for restriction endonucleases that do not cut HSV-1 DNA, the *pac* signal can be deleted, and the recombinant genomes can be reintroduced into *E. coli* as BACs to serve as helper DNA for the packaging of amplicons into HSV-1 virions. The strength of the new packaging system is the simplicity: cotransfection of the amplicon plasmid with a single helper BAC plasmid, rather than with five cosmids; the high genomic stability of the BACs, whereas some of the cosmids have proven unstable; and the ease of introducing into the BACs mutations that will decrease cytotoxicity, and hopefully increase titers, as compared with more complicated manipulations required for the cosmids.

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